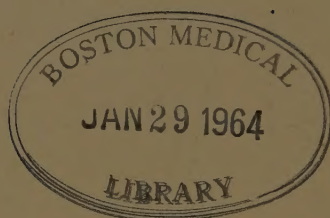


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MECHANISM OF ENZYME ACTION AND
ASSOCIATED CELL PHENOMENA

MECHANISM OF ENZYME ACTION
AND
ASSOCIATED CELL PHENOMENA

BY

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"It seems to me that perhaps the only advantage of advancing age is that one is able to recall what the old masters thought."

—GRAHAM LUSK

PREFACE

Intimation has been given by several workers of the lack of a short, disinterested orientation on certain sections of enzyme chemistry which could serve as a preliminary insight for those who are in search of detailed information. It has only been with hesitation that I have summarized, therefore, my recent lectures before American Chemists and at several Japanese and Indian Universities in the hope that this endeavor to clear up at least certain points of earlier representations in this field might contribute toward the purpose.

The expression of my obligations is due to Dr. R. A. Gortner, of the University of Minnesota, for many discussions, to Mr. Alan E. Treloar, B. Sc. Agr., of Sydney, Australia, for his kind syntactic assistance and to Mr. J. Weichherz, D.E., of Berlin for reading the proof.

F. F. NORD

Berlin, March, 1928

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CHAPTER I

INTRODUCTION

One of the most important ways in which the products of agriculture are used is the utilization of carbohydrates as raw materials for the various fermentations, alcoholic, lactic acid, butyric acid, acetic acid and tobacco (46), brought about by microorganisms. We know what the original materials and end products are in these biochemical reactions, but we are just in the development of a period whose task it is to penetrate the mechanism of these reactions by use of the tools of biochemistry.

The very great importance and significance of investigations concerning the transformations of carbohydrates and the relation of those transformations to biological science can be indicated by reference to a few facts. The carbohydrates are formed in the assimilation processes of plants and serve as the starting product for the chemical and physical performances of work by the plants. Further, nearly all bacteria take up some form of sugar from their nutrient media. Not only that, but in the animal body carbohydrates can be responsible for the synthesis of protein and fat.

All are familiar with the experiment of two naturalists performed some sixty years ago (44). They climbed the Faulhorn in Switzerland, a peak 1956 meters high. Seventeen hours before starting they ate the last nitrogenous food taken during the experiment. During eight hours they climbed with but one interruption, and were in motion a total of thirteen hours. They carefully collected the urine excreted during that time and showed, by its analysis, that the work

done corresponded to three times the energy which could be derived from the protein used. This experiment was the starting point of all observations which have shown that, in the case of animals as well as of other more highly developed organisms, the source of energy is not in protein but primarily in fat and carbohydrate.

Workers in this field have shown that the carbohydrates are thereby, in general, burned to carbon dioxide and water. But the yeasts, even in case of increased aeration with oxygen (49), use less sugar material for alcohol production than they have at their disposal for this purpose. This shows that in this process there is at least a partial resynthesis of sugar from the intermediate products. We also know from numerous observations made on various organisms that in this disintegration, analogous to purely chemical oxidation, there must be intermediate stages.

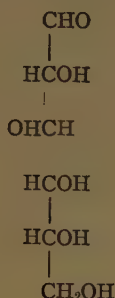
Excluding, obviously, the case of a biological oxidation, if we are inclined to consider fermentation as a process through which, by a biochemical action, carbon chains are broken down or united (see particularly pages 39, 61), it is intelligible that for many decades the efforts of naturalists have been directed toward an interpretation of the decomposition of sugar, using as an example the long known phenomenon of alcoholic fermentation, a process typical of one that stops, to a certain degree, at an intermediate stage.

The decomposition of carbohydrate by yeast was expressed, in its quantitative proportions, by the equation of Gay-Lussac (50):

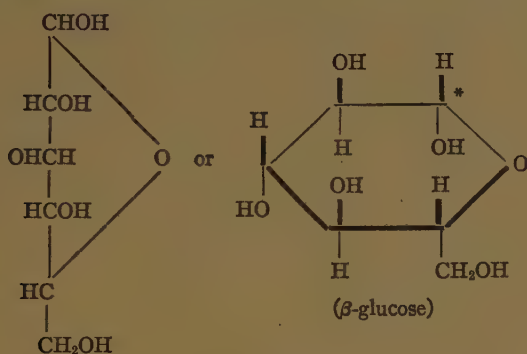


We cannot simply interpret these proportions. The practical quantitative course of the fermentation process produces

from each mol of sugar two mols each of alcohol and carbon dioxide. The old familiar formula of glucose,



and the newer one suggested by Haworth (64)



* Reducing group.

give no indication at all of the ethylidene or carbon dioxide groups which occur in the fermentation products. Here, then, is a typical example in which the final condition comes about only through intermediate steps, a process which involves a gradual dissolution of the hexose carbon chain.

The decomposition of fermentable initial materials in the course of enzymatic alcohol production seems to rest, in the

ultimate analysis, on the fact that the substances formed in the course of the reaction are easily convertible and difficult to intercept. These substances must, however, in every case, be such that the yeast can further act upon them and finally render possible the production of compounds which are in equilibrium with each other and which give the end products of the fermentation.

CHAPTER II

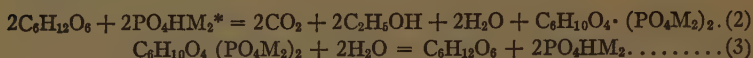
THE RÔLE OF ZYMOPHOSPHATES

In regard to the first phase in the decomposition of the sugar complex, i.e., the processes which probably initiate the disintegration of the hexose molecule to substances containing three carbon atoms by the action of the yeast enzyme it is difficult to reach any clear cut conclusions from the existing experimental data. It is, however, supposed that the alkali phosphates have an important rôle in this phase.

The first proposal for the use of phosphates, namely, to add to a 15 per cent fermentable sugar solution 0.2 gram per liter of monocalcium phosphate, came from Pasteur (147). After Buchner and Hahn (15) had ascribed initial acceleration of fermentation by alkali phosphate to the alkaline reaction of these salts, L. Iwanow (74) starting in 1905 and Harden and Young (56), began fundamental investigations in connection with this question. The British investigators noted that the speed¹ of the fermentation increased if one added, in the presence of phosphates, boiled or ultrafiltered yeast juice. This action apparently should be ascribed to the phosphates, as similar observations were made when solutions of the alkali salts of orthophosphoric acid were used. At the end of the fermentation, no phosphate could be detected in the usual way. It might be supposed, on the basis of later investigations, that in the alcoholic fermentation two molecules of sugar are always

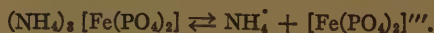
¹ According to Slator (Chem. Soc., 89, 133, (1906)), the speed of fermentation is directly proportional to the quantity of yeast and allegedly only very slightly dependent on the sugar concentration between 0.5 and 10 per cent, but it may be diminished by the simple fatty acids. (H. Katagiri: Biochem. J., 20, 427; 1926; 21, 494; 1927.)

concerned, of which one combines with two molecules of phosphate to form hexosediphosphoric acid while the other molecule of sugar breaks up to form carbon dioxide and alcohol. In the further course of the reaction a special enzyme, the phosphatase, continually breaks down the hexosediphosphoric acid to inorganic phosphate and fermentable hexose, whereby the repetition of the process becomes possible:



*M = metal

Previously, however, Harden and Young, by means of kinetic measurement and analysis, had made the important observation that the increase in the amount of carbon dioxide or alcohol formed was, within definite limits, directly proportional to the amount of phosphate added (see fig. 1). In the graph, curve A shows the normal course of fermentation with yeast juice alone (l.c., p. 416), curve B the effect of adding phosphate. In the latter case the velocity of fermentation amounted to 9.5 cc. per five minutes, i.e., some six times the normal value, and then again reached almost exactly the original value of 1.4. Curve C shows the repetition of the whole phenomenon after a period of 70 minutes when the phosphate was renewed. Recent experiments by Lyon (97) seem to show that the active component of phosphate solutions, in relation to promoter action on oxidizing enzymes, is the PO_4 ion, the effect of which, however, may be checked according to Hodel and Neuenschwander (71) by the ferric ion through the formation of ferric phosphate:



Recently, Meyerhof (103) suggested a somewhat different interpretation of the equations of Harden and Young. He

believes that the processes proceed in two phases. In the first of these the formation of two molecules of an "active" hexose-monophosphoric acid is supposed to occur. One of the latter molecules splits during the *status nascens*, whereas the second

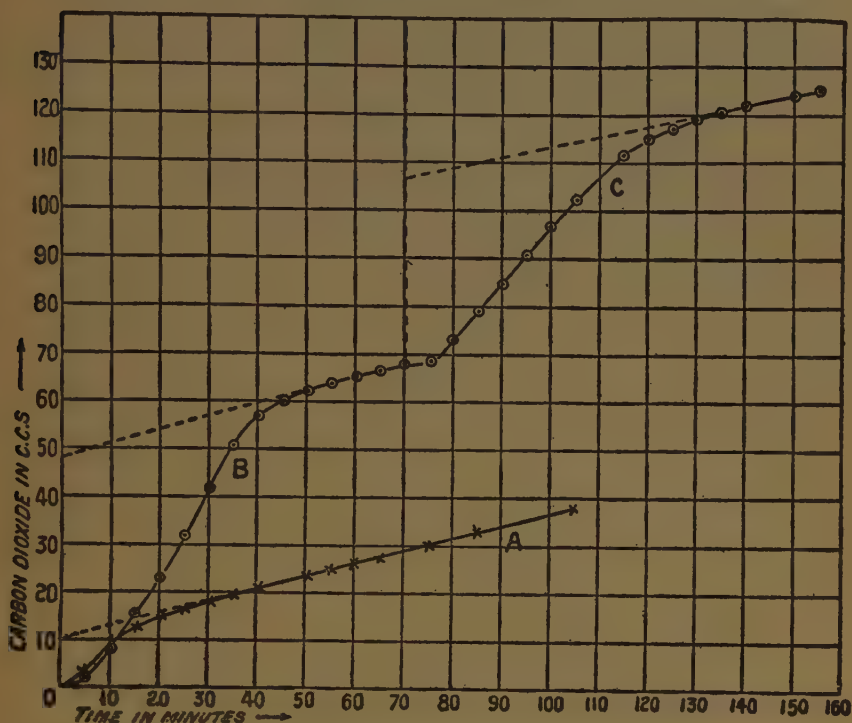


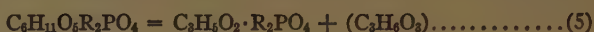
FIG. 1

forms the stable hexosediphosphate. The slow but direct fermentation of the hexose-diphosphate allegedly takes place in the second phase. This consideration tacitly adopts the suggestion of Komatsu and Nodzu (83,127) and the conception of Raymond (152), the latter assuming that the

hexose-mono-phosphoric acid of Robison (157)² is the intermediate conversion to the ester of hexose-di-phosphoric acid. This takes place through the hexose-mono-phosphoric acid:



which splits into a phosphorus-containing and a phosphorus-free triose:



the former condensing to hexose-di-phosphoric acid,



and the latter breaking down to alcohol and carbonic acid,



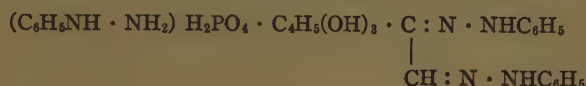
It is noteworthy in both propositions that neither is the postulation of "active" monophosphates explained, nor is the assumption of a particular reactive triose adequately supported by experiments.³ Moreover, they fail completely to give an account of the processes which are doubtlessly indispensable in explaining the physico-chemical mechanism which is involved in the living cell.

The organic phosphoric acid compound, which is not precipitated by magnesia mixture, was isolated by L. Iwanow as the copper salt, in 1905; it can also be precipitated by lead acetate. This Russian investigator considered it a triose derivative having the formula: $C_3H_5O_2 \cdot H_2PO_4$ and later showed that

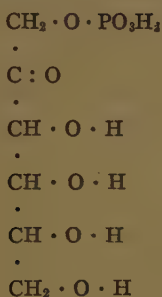
² Which occurs together with hexosediphosphate but is not identical with the decomposition product of Neuberg.

³ Compare also A. J. Kluyver and A. P. Struyk (Naturwissenschaften, 14, 882; 1926). The recently published experimental results of the authors (Proc. Acad. Sci. Amsterdam 30, 871; 1927) failed, however, to give a convincing evidence for this deduction.

this change of inorganic phosphate to organic compounds could be brought about by many of the higher plants. Lebedew (91), assuming he was dealing with a hexose-mono-phosphoric acid, prepared a phenyl hydrazine derivative of the supposed formula $C_{24}H_{31}N_6O_7P$, but again Harden and Young (57) were able to produce conclusive proof that the substance was the phenyl hydrazine salt of a hexosephosphoric acid osazone having the formula:



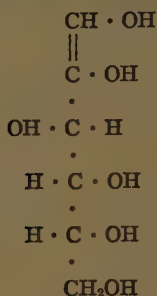
That the carbohydrate phosphoric ester is, nevertheless, really a hexosediphosphate:



(where we leave open the question which of the H-atoms is replaced by the second phosphoric acid radical)* was established by Young (58) when he showed that in the reaction with phenylhydrazine there occurred a separation of one mol of phosphoric acid. Also, the later degradation to the hexose-mono-phosphoric ester: $C_6H_{11}O_5 \cdot H_2PO_4$ by Neuberg (111),

* Note added at correction: According to the recent findings of P. A. Levene and A. L. Raymond (J. Biolog. Chem. 80, 633; 1928) the stable phosphoric acid residue of the fructose diphosphate of fermentation is attached to carbon atom (6).

can be viewed as further clarifying the nature of the diphosphates of d-glucose, d-fructose, and d-mannose (corresponding zymophosphates); but we do not know with much precision, as yet, the configuration of the hexose present in the ester. The fact that the γ -fructose present in cane sugar is partially enolized certainly suggests the possibility of the enol formula:



The possibility of a re-formation into fructose as shown by equation (3) obliges us, even if we accept the view of Harden and Young, to consider that the hexose-diphosphate is not a decomposition product but an intermediate compound still retaining six carbon atoms. This conception is supported by the findings of L. Iwanow, v. Euler, and Johansson, who have shown that the formation of hexose-diphosphate can take place independently of fermentation. v. Euler, Kullberg, and Olsen (35) ascribed this effect to an enzyme which does not act reversibly. This enzyme may be identical with the synthase earlier assumed by Iwanow. Differences of great magnitude exist between the two conceptions. The significance of phosphate esters in the course of alcoholic fermentation, in contrast with the change of substances in muscles, i.e., the formation of the myophosphates (28) thus remains as yet obscure, (unless we be permitted to predict a conciliation of the contradictions in a manner analogous, perchance, to the findings (186) according to which the fact of fermentation

of *composite* sugars by the effect to specific zymases without⁴ preliminary hydrolysis is due to a considerably greater number of zymases than as hitherto supposed).

The grounds for this are as follows: It is accepted as proven that zymophosphates are, in general, unfermentable by living yeast,⁵ and also that phosphoric esters can be obtained only by means of yeast juice or dried yeast. If, then, the formation of the phosphoric ester be possible without fermentation, it remains questionable whether the two phenomena are to be considered inter-dependent and whether suitable conclusions may be drawn from the proportion between the fermented and phosphorated sugar.

As a confirmation of these scruples could be regarded the recent findings of Harden and Henley (62) and might justify again the consideration that the mode of acting of the phosphates during the course of fermentation may be compared also with that of a buffer system, thus enabling the enzymes to maintain more or less permanently that pH which we know is prevalent at the place of enzymatic activity within these cells, i.e., *ca* pH 6.0 (172).

If we further consider that an accumulation of hexose-diphosphate takes place only in the presence of an unusually large amount of salts of phosphoric-acid (as well as in the case of one of the normal alcoholic fermentations having a less

⁴ The probability of this interpretation was recently denied by R. Cohn, (Z. physiol. Chem., 168, 92-116; 1927), and this denial is in good agreement with the findings of Somogyi (167) who has shown that the fermentable sugars are in contradistinction f.i. to lactose, arabinose, etc. adsorbed by the yeasts.

⁵ This was recently conclusively confirmed by Nord and Franke (J. biolog. Chem., 79, 27; 1928). The amount of CO₂ obtained through the "fermentation" of an original preparation from Elberfeld was only equivalent to the quantity of unbound sugar present there and corresponded with the analysis communicated by Dr. Hörlein.

favorable hydrogen ion concentration, $\text{pH} = 6.4$),⁶ then it is interesting to note the opinion that we perhaps have no longer to deal with the usual fermentation process but with an abnormal one which will be discussed later—one which is similar to the peculiar general kind of forced glycerol fermentations.

But these considerations of Neuberg, Faerber, Levite and Schwenk (117), repeatedly referred to since 1917, seem again to require revision in view of the results of experiments by Smedley MacLean and Hoffert (164).⁷ The experiments of these British investigators indicate that the larger hexose phosphate molecule cannot permeate the wall of the yeast cell, but that the sugar and phosphate enter the cell separately and then combine. Since yeast contains one of the enzymes which brings about the synthesis of hexosephosphate, which allegedly does not pass through the cell wall, it seems to be easily intelligible that the hexosephosphates may be demonstrated only in cell-free fermentation after the enzyme is present in the fluid.

Probably assuming that the combination of the hexoses with inorganic salts, which is supposed to initiate the real decomposition of the sugar molecules, takes place outside of the cell, Paine (144), in the laboratory of Harden, investigated the permeability of yeast cells to hexosephosphates. These experiments have been interpreted very differently (80) by

⁶ According to Haegglund and Augustson (*Biochem. Z.*, 155, 334, (1925)) the highest fermentative activity of living yeast is attained at $\text{pH} = 4.5$. This appears to be too low, in consequence of the recent observations of Nord and Franke (*Protoplasma*, 4, No. 4; 1928). Compare also M. M. H. Van Laer (*Bull. Soc. R. Medic. Nat. Bruxelles*, 1925, p. 46).

⁷ It is probably through an error that M. Schoen (*Monogr. de l'Institut Pasteur*, No. 3, p. 128: 1926) recently ascribed this suggestion to other authors.

different workers and in some cases very strangely. According to Harden, "the yeast cell is at all events partially permeable to the sodium salt." Höber draws the conclusion that the permeability to salts is small, but he regards it also as possible that the whole phenomenon may simulate superficial adsorption. Finally C. Neuberg understood from the description of the experiments that the cell is "durchaus," permeable to hexose-di-phosphoric salts.

The above mentioned assertion (164) might be regarded as an unconscious application of the proposition of Ruhland and Hoffmann (159), wherein the smaller the volume of the molecules, the faster is their penetration into plant cells supposed to take place. In spite of the fact that this is contradicted by the rule of Overton, the assertion possesses a certain probability (128).

We must remember that the almost impossible detection of hexosephosphates in fermentations by means of yeast cells, is in good harmony with the abundant formation of hexosephosphates by fermentations which are free of cells. The membrane of the cell is scarcely permeable to the synthease of Iwanow. With uninjured yeast cells there is in the outer medium only a very small quantity of hexose-di-phosphate which might partially penetrate into the cell. If we henceforth assume, more especially in accordance with the considerations and experiments of Witzemann, Gurchot and others, that the membrane of the yeast cell also represents a dynamic system⁸ which might be compared to a copperferrocyanide membrane, and therefore can be acted upon by intermittent coagulation \leftrightarrow peptization, then the whole process will become readily intelligible.

⁸ Similar conclusions, in so far as the processes involved in the contraction of the muscle are concerned, were reached by G. Embden and H. Jost (Z. physiolog. Ch., 165, 225; 1927).

The externally produced hexose-di-phosphates penetrate into the interior of the cell until a suitable salt concentration is reached which brings about the coagulation of the membrane. Through the fissures of this now "crystalline" membrane, uncombined sugar—which is typically non-diffusible through a truly semipermeable membrane⁹—can now penetrate into the cell where it will be esterified by means of the endocellular synthease. The alteration (not fermentation) of the hexosephosphates into a "transportation form" of the sugar is postulated to take place isochronously. This latter is again subject to direct splitting into the compounds of the three-carbon chain, changing the internal concentration of the salts in such a manner that a reeptization takes place, the influx of the sugar ceases, and the cycle may be started again.¹⁰

The chief characteristic of the process would be, under these conditions, an intermittent coagulation-peptization of the membrane as well as endeavoring to maintain a membrane equilibrium in the sense of Clowes (17).¹¹

It is highly probable that the greater part of the sugars is esterified within the cells where the enzymes exerting fermentation are located and where they will be liberated. It is very important, therefore, to have a conception of the mechanism of the admittance. In contrast to this, it is only of secondary significance whether the hexose-di-phosphates originate through an intermediate hexose-mono-phosphate. In any case, they are disintegrated and leave behind the sugar in the

⁹ Compare also Ferguson, J. Chem. Soc., 6, 122; 1854.

¹⁰ Compare G. Bredig and M. Minaeff, *Festschrift z. Hundertj. d. Technischen Hochschule Karlsruhe*, 1925.

¹¹ No acceptance is expressed, herewith, of his or v. Möllendorff's (1918) opinion that the membrane is comparable to an emulsion, which, of course, would make it impossible to understand the osmotic activities of the cell.

transportation form, which is readily cleavable and which does *not* (104) need to be re-esterified. Isochronous rearrangements in intermittent processes exclude, of course, the accumulation of any intermediate products in normal fermentation.

The results of measurements made by v. Euler and Nilsson (36), are of great importance in understanding the introduction of the reactions in the three carbon chain series (see Chapter VI). These investigators in logical valuations of the fundamental investigations of Witzemann (188), as well as of Spoehr (169), and in agreement with Kuhn and Jakob (86), apparently have established that in the case of non-enzymatic reactions the reactivity of the zymohexose molecule, especially in the case of fructose, is essentially raised.

This fact agrees with the important observations of Warburg and Yabusoe (182). According to them there is indeed combustion of fructose in the presence of phosphate ions and molecular oxygen, as opposed to glucose, which is not affected. It would be attractive, on the other hand, if we could possibly regard an ester of a γ -sugar (the "transport form" of the dextrose of Hewitt and Pryde (66), the structure of which is still unknown) as being the result of the action of the Synthease (see before), instead of on the fructose-di-phosphate. It will be especially necessary, in agreement with Irvine (73), to suppose that even in enzymatic processes primary steps of isolated sugars are not chemical individuals but labile forms.

The chief characteristic of the transportation form (129) of a compound or system may be regarded as its capability either to mediate in intermittent actions, or to enable irreversible reactions to proceed, especially in cases where the use of a potentially higher energy content is involved.¹² It is not sup-

¹² It is under these circumstances misleading when, the hexose-mono-phosphoric acids are designated to be "active" (compare p. 8) as has recently been done.

posed to exist in a form which can be directly investigated successfully as a chemical entity by any means at present at our disposal.¹³ Its capacity to promote the aforementioned types of biological reactions is probably due, in the main, to an electron transfer caused by the ionic antagonism within the cell. Ionic antagonism, we know, exerts a great influence upon enzyme action and there are certain reasons for assuming that the same is also true of the influence of adsorption.

¹³ See also S. A. Schou and R. Wurmser, *Comptes rendus*, **186**, 369 (1928).

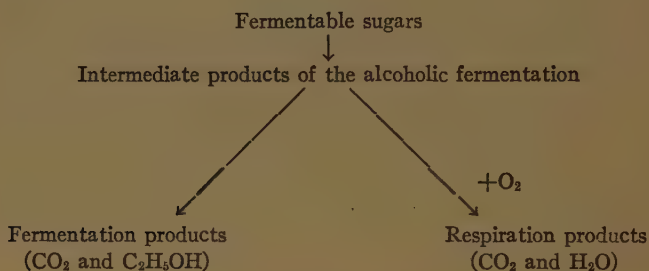
CHAPTER III

THE CO-ENZYME

For the initiation of fermentation or of phosphoration, not only are enzymes and mineral phosphates necessary, but also auxiliary systems. Harden and Young (l.c.) in 1906 let yeast juice (from top yeast) pass through a Martin gelatin filter and thereby obtained an inert residue and inert filtrate. Buchner and Antoni (14) dialyzed the juice through parchment paper and obtained the same results. If residue and filtrate, each of which can no longer decompose sugar, be united, then one obtains a mixture which is equal to the undialyzed juice in its ability to bring about alcoholic fermentation. Besides the zymases a system which is dialyzable and, to a certain extent thermostable up to 80° according to Tholin (173) is also necessary for the fermentation. Following the suggestion of Bertrand, Harden and Young called this system a co-enzyme (59). The inactive residue from the dialysis can be made active again also by the addition of boiled juice or by the addition of an inactive yeast extract termed also apozymase. The complex zymases capable of bringing about fermentation are composed, then, of the sum of the co-ferments plus apozymase, hence the former is one part of an effective system of ferments.

Meyerhof's observation that the co-enzyme of alcoholic fermentations, the molecular weight of which is stated to amount (37) to 486 ± 6 , occurs in the muscles and organs of animals as well as in milk (105) is of significance in this regard. According to Virtanen and Simola (179) it can be also found in blood corpuscles. Meyerhof also showed that aqueous extracts of animal muscles and germinating plants strongly furthered alcoholic fermentation. This seems to be entirely in accord with the earlier observation of Kostytschew, Hüb-

benet and Scheloumow (84), that yeast extract increases the normal respiration of plants. Meyerhof, on this basis, suggested that the same co-ferments are necessary for the normal respiration of animal tissue as for yeast fermentation, i.e., it was supposed that the hexosephosphate was just as indispensable for the fermentative oxygen respiration of animals as for alcoholic fermentation. Although Scandinavian investigators have concentrated the co-ferment by precipitation on tannin, phosphotungstic and silicotungstic acids (38), the character of the system remains poorly known. Among all the different interpretations, that of Meyerhof is to be noted wherein he suggests the probability that a connection exists between fermentation and respiration. This view can be shown schematically, according to Kostytshew (l.c.), as follows:



It is finally based on the classical hypothesis of Palladin (145) that the oxidation and reduction ferments of vegetable tissue transform the primary products of sugar splitting, previously formed by the enzymes of fermentation, to the end-products.

Although v. Szent Gyoergyi (171), for example, has stated that he was able to replace co-ferments by definite substances (p-phenylenediamine), we are unable, in view of the present situation in the field of the "kinases," to state definitely whether the systems in question represent a real auxiliary catalyzer. (Compare also page 40.)

CHAPTER IV

A THEORY TO INTERPRET THE SO-CALLED "ACTIVATION"

"And yet after reading of him in scores of volumes, and hunting him through old magazines and newspapers, having him here at a ball, there at a public dinner, there at races and so forth, you find you have nothing—nothing but a coat and wig and a mask smiling below it—nothing but a great simulacrum . . . I look through all his life and find but a bow and a grin—I try and take him to pieces, and find silk stockings, padding stays, a coat with frogs and a fur collar, a star and a ribbon, a pocket handkerchief prodigiously scented, one of Truefitt's best nutty-brown wigs reeking with oil, a set of teeth and a huge black smock under-waistcoat, more under-waistcoats and under that nothing."—THACKERAY, on George IV of England.

For a long time two viewpoints regarding the mechanism of enzymatic activity have profoundly influenced our conceptions in this field. To Oscar Loew is due the credit for the original suggestion that the enzymes possessing atomic groups with kinetic lability are able, even at a comparatively low temperature, to perform chemical action. This suggestion was later modified and we now generally assume that, as in the case of heterogeneous reactions, the reactants are adsorbed by the enzyme in order that reaction may ensue. In accordance with this it might be accepted with Bayliss, that the reaction velocity is determined by the concentration of adsorption complex, i.e., reactant-enzyme, present in the system. The possibility of carrying out such reactions doubtlessly depends on certain conditions of the surface (130), and is in general influenced also by the hydrogen ion concentration.

In order to perform suitable experiments, Nord and Franke (139) found it necessary to prepare zymase solutions which

behave as lyophile colloids. Lebedew (88a) was among the first men to use such solutions. There is a widespread belief that one of the chief characteristics of such zymase preparations is that they lose their activity after twenty-four hours, or at 0°C. within two days. C. Oppenheimer (143) states:

“Zymase verliert in Loesung nach 24 Stunden, bei 0° in 2 Tagen ihre Wirksamkeit, was vor allem auf die schaedliche Wirkung der Hefenprotease zurueckzufuehren ist.”

After having prepared similar solutions from American bottom yeasts belonging in the group of *Saccharomycetaceae* of the second sub-group in the Hansen-Guilliermond classification, Nord and Franke found that this statement was essentially in disagreement with their observations. The solutions of these preparations were absolutely cell-free,¹ and contained probably much less of the zymase-destroying yeast protease. The solutions were found to be not only capable of exerting their full activity even after preservation for two months at -5° to -15°C., but developed within the first few days of this preservation an added activity which doubtlessly would always show a measurable increase of carbon dioxide production due to an increased surface, if it were not necessary to take into consideration the possibility of a simultaneous disaggregation of the zymase by the protease which is present. In spite of the investigations of Dernby (22) or Willstätter and Grassmann (187) it is difficult to determine the extent of this loss of zymase activity because of the uncertainty of our knowledge concerning the performance of protease action in relation to temperature and time.

This change of activity manifested itself in gradually de-

¹ The considerations of Kostytschew, Medwedew and Kardo-Sysowewa (*Z. physiolog. Chemie*, **168**, 255; 1927) are in view of our clear experiments, on account of their backwardness, highly deplorable.

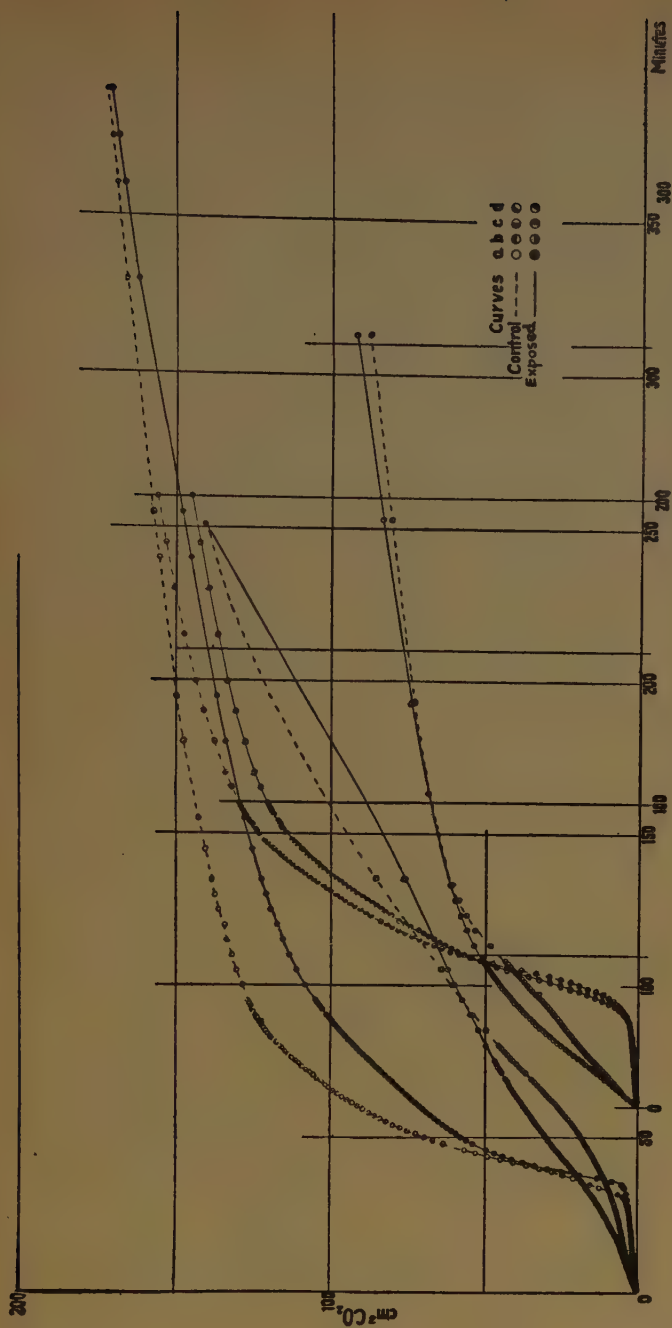


FIG. 2. Influence of a protector upon zymase activity. (a) 1 gram of glucose was fermented with 20 cc. fresh juice exposed for twenty minutes to ethylene. (b) Fermentation of an additional gram of glucose. (c, d) Repetition of the experiment after twenty-four hours.

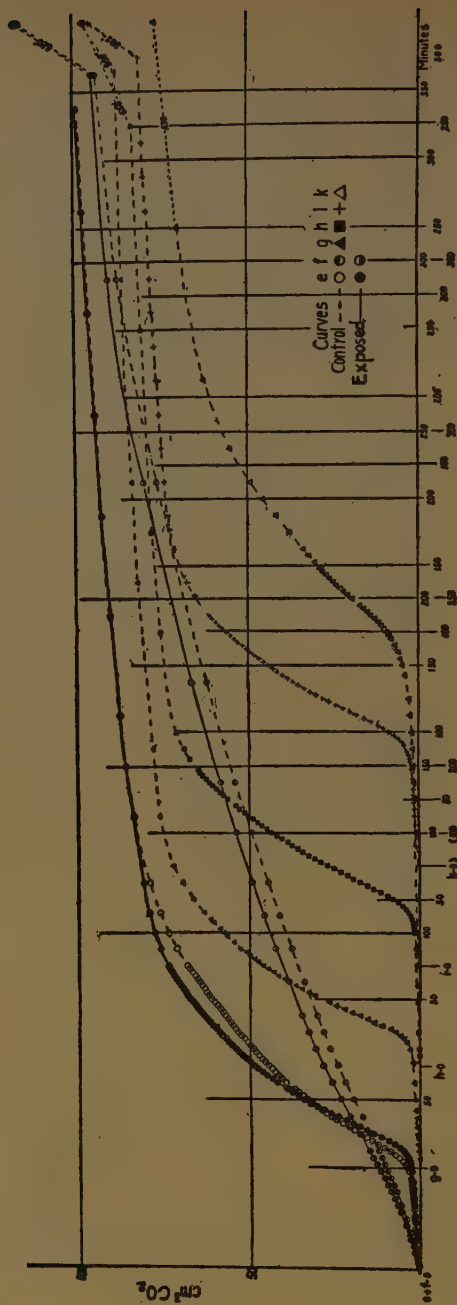


FIG. 3. Influence of increased peptization in the presence or absence of a protector. (e.f.) 0.5 gram of glucose was fermented with 10 cc. juice after 3, (g) 5, (h) 15, (i) 28 or (k) 57 days.

creasing viscosity or increasing surface tension. The change of both properties reaches a certain minimum or maximum respectively after a few days. The increased fermentation activity on the one hand and the corresponding change of physico-chemical properties on the other hand are in good agreement with the general behavior of a lyophile colloid, and indicate clearly that the peptization of the colloid enzymes

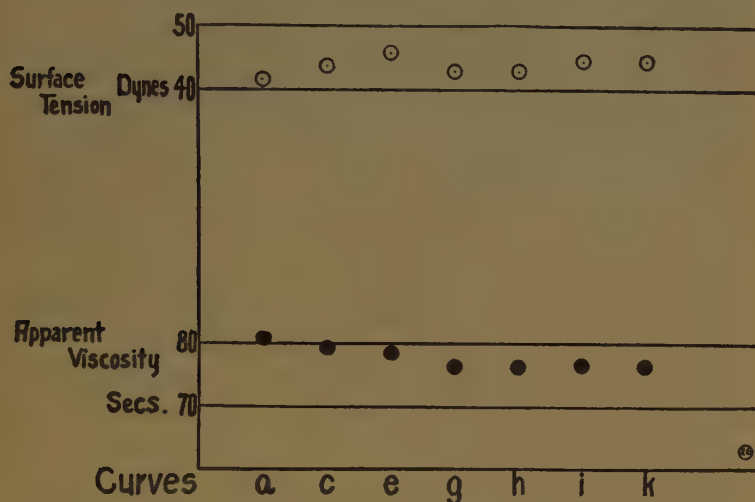


FIG. 4. Graph representing the corresponding change of surface tension or apparent viscosity of the zymase solution used in the experiments pictured in figs. 2 and 3.

concerned becomes greater, causing an appreciable increase of their surface.

Experiments of Harden and Young have given indication that the velocity of fermentation carried out with zymase solutions prepared after the original method of Lebedew remains approximately constant until a certain very low limit of sugar concentration is reached, and then falls rapidly. It

was very remarkable to observe in our experiments (Nord and Franke (139)) that the speed of fermentation, measured on the basis of carbon dioxide production in a unit of time, after passing a certain induction period, reached an extraordinarily high value, which was maintained only for a short time, decreasing to a value which was usually measurable—but which nevertheless could not be explained as being due to the lack of fermentable sugar. It appears justifiable to interpret this observation as a further confirmation of an increased surface of the enzyme concerned, indicating at the same time that this increased surface is apparently much more sensitive toward the products of the yeast metabolism. It might therefore be regarded as correct to assume that there is always a certain concentration of enzymes present which is potentially capable of action. However, the reactivity of the enzyme is dependent on its surface activity. Immediately after the initial reaction, the surface undergoes alterations which relatively decrease the velocity of the reaction, independently of the concentration of the reactant. In the course of the reaction the ratio between active and “damaged” enzyme may decrease more and more below unity.

Recently Gortner (51), without referring to any special case, postulated that the forces which are operating on the surface are purely physical in certain instances, while in other cases they are due to the forces of chemical valence. In contra-distinction to this P. Pfeiffer (149) felt prompted to re-emphasize that the associations between enzymes and the substrata belong in the group of true molecular combinations, i.e., are due to chemisorption. If the latter could be true, it seems impossible to interpret in a proper manner the observations mentioned above, supplemented as they are by those which are to follow.

The comparatively rapid decrease of the speed of reactivity

in the colloidal zymase solutions, of which the degree of peptization was increased, prompted Nord and Franke to look for means to maintain the surface conditions of the zymases concerned. It was thought advisable for this purpose to use certain gases in such minute quantities as to exclude *a priori* the thought that they could also be acted upon in the course of the reaction between enzyme and reactant in such a manner that a significant *chemical* reaction could be co-responsible for the resultant observations. In connection with this it has to be mentioned that Levy (93), proceeding from the experiments of Effront (26) on the action of abietic acid on fermenting yeasts, was the first to regard such occurrences as an "activation of ferments." Using zymase solutions several years later, Cassel, Euler, M. Oppenheimer and others rediscovered this observation, and it was then postulated that certain substances, especially aldehydes, have a decided effect of activation on alcoholic fermentation. However, no definition for in this respect mysterious conception was ever given and it was only because of the lack of objections founded on experimental evidence that the repeatedly introduced proposition which connected the so-called activation with reducibility was tentatively and hesitatingly accepted (11). The need of a satisfactory explanation of its origin was clearly felt, for example, by Soda (166), who was induced to investigate the effect of different substances chosen at random, but who admitted that "Hier koennen vorlaeufig nur die Tatsachen registriert werden." The uncertainty regarding the real meaning of the "activation" has not been lessened by a recent work of Mameli (101), who offers certain data but gives no explanation.

By exposing enzyme solutions to certain gases it was then found that when these came in contact with the enzyme sur-

faces, which did or did not possess increased reactivity due to an increased peptization, the adsorption took place in such a manner as to appear to be due to the formation of a protective surface, especially in view of the above mentioned improbability of chemical action. They neither increased nor decreased the induction period of the fermentation process. It is suggested that this property of substances be referred to as *protector action*. By using such protectors it was possible to delay the speed of the reaction reflected in the change of the quotient noted above (see page 24). Since the most favorable conditions for the performance of an enzymatic reaction are in many cases not known, the statements above suggested the conclusion that in a great number of so-called "activations" of enzymatic processes by influencing those reactions through chemical compounds, no activation in fact takes place but the so-called activators, which appear to be really protectors, enable the enzymes to act for a prolonged time under conditions which are of more near approach to the ideal. The use of certain gases offered the great advantage of being sure that when influencing the course of the reaction the investigators were practically independent of the gaseous concentration within the meshes and were only dependent upon the changed surface being produced on the enzyme, regardless of whether the enzyme was free or whether it was still bound to the living cell. This conclusion applies even more to liquids or solids introduced into similar media and explains at once their unsatisfactory and uncertain manner of action.

Our assumptions were supported by the following considerations and experiments insofar as the use particularly of ethylene and related gases is concerned:

1. The real existence of two types of double bonds was experimentally proven by Sugden, Reed and Wilkins (170).

This enables us to assume that in the condition of adsorption the opposed charges



are neutralized in contradistinction to the stationary situation when the condition appeared to be reflected by this formula



2. "All film-coated surface regions are," according to Ramsden (151), "inherently contractile, even when the coating is itself rigid, and when given the opportunity to contract will pile up the coating into visible masses, or if soluble, allow it to pass back into solution."

3. From data of Neljubow (108) it is known that ethylene exerts an influence on geotropy of stalks.

4. Experiments of Edward Maris Harvey (63) convey the impression that ethylene was found to be very effective in producing changes in general processes of plant metabolism. These observations were later extended by Denny (21).

For a long time it was thought proven that one of the main factors of the effect of small quantities of certain substances in the course of certain enzymatic reactions, e.g., alcoholic fermentation, was due to the fact that they may be reduced. Even E. Beckmann apparently did not criticise various data which were supposed to support this belief, in spite of the fact that the experiments concerned were carried out in solutions which were not previously saturated with carbon dioxide; thus rendering unsteady the control of factors governing the retention of the carbon dioxide evolved under the conditions of the experiment. The term of activation, especially in connection

with these enzymatic reactions, has been and still is used with such a hazy meaning that it has delayed in an extensive manner the clarification of our conceptions, and in reality has not decreased the prevalent uncertainty, in one of the most important fields of biological chemistry.

The interpretation of all the factors becomes much more intelligible by applying the capillary theory, assuming simultaneously that there is the promise of further experiments bringing perhaps conclusive evidence that adsorbed vapor films are monomolecular in thickness. In this way we would have films of ethylene for instance, or of other polar compounds, which convey the possibility of the neutralization, or in any event the alteration, of the opposed charges (see page 27) on the surface of the colloidal enzymes, enabling the latter to act freely upon the substrate, and at the same time protecting them from the possible effect of transformation products.

In order to prove this hypothesis it was deemed suitable for the purpose not only to work with enzyme solutions, but to investigate also the effect of bi-polar molecules upon enzymes within living single cells and cells of tissues as for instance, leaves, all the more so since it is generally accepted that the influence of a given hydrogen ion concentration on the activity of enzymes is very pronounced. However in the case of yeasts several series of experiments were carried out taking into consideration the very wide ranges of hydrogen ion concentration within which they are capable of acting. These experiments indicated, in fact, that perhaps adsorption plays a greater rôle in influencing the enzymatic activity within certain hydrogen ion concentration ranges than has been generally admitted up to the present.

In comparing the observations of the subsequent experiments with the experiments carried out on enzyme solutions,

however, it was very surprising to see that in the case of cells the effect of a bi-polar molecule, exerted in the form of a surface film, is preceded by a decided effect upon the permeability of the cell itself. Luckhardt and Carter (98) published the first observations upon the narcotic effect of ethylene, and it should have been expected that, in accordance with the general belief that in the condition of narcosis the permeability of the protoplasm is decreased, the same should have been observed here also. Instead of this the opposite appears to be true. By exposing a suspension of yeast cells to a slowly bubbling current of ethylene gas and subsequently adding glucose to be acted upon by those cells, it was observed that a far more rapid fermentation took place in the beginning than was the case with an unexposed yeast suspension. However, during the course of the experiments, the observation was very often made that the exposed suspension fermented even more slowly for a certain period than the unexposed suspension. After a new addition of sugar into the fermenting mash, which, of course, contained also the main metabolic product of the fermentation, i.e., alcohol, the exposed solution not only fermented a greater part of the sugar available, but even the carbon dioxide quantity produced in the unit of time was greater. The logical consequence of this observation was to include the possibility that under the effect of ethylene, there was a more rapid multiplication of yeast cells. In order to exclude this uncertainty, not only were cell counts made after terminating a certain fermentation, but also fermentations were carried out with certain zymase preparations, which allegedly represent killed yeast cells which still maintain their fermenting capacity. However, the earlier observations both on the cells and on zymase solutions were confirmed. It was difficult to bring our observations into harmony with the generally accepted belief that the effect of narcotics on cer-

tain cells decreased their permeability. An intensive search of the literature did not disclose any similar observations. While these investigations were being conducted, however, a comprehensive paper by Hoefler and Weber (72) was found, and in the discussion of their experiments on the effect of ether narcosis upon the permeability of plant cells for urea, they reached the conclusion that the general proposition that narcosis decreases the permeability of protoplasma had to be abandoned.

The experiments of Nord and Franke collaborate this conclusion, but only on the basis of colloid chemical theories of the plasm structure, more especially if the consideration explained before be accepted, namely that the membrane of the yeast cell represents a dynamic system which might be compared to a copper ferrocyanide membrane and, therefore, can be acted upon by intermittent coagulation \rightleftharpoons peptization.

Experiments carried out with enzyme solutions, or yeast cells, or tobacco leaves (140) (in order to study the oxygen evolution by means of catalase) indicate clearly that zymase solutions exposed to ethylene fail to show a measurably increased production of carbon dioxide at the beginning of the course of the fermentation. This may be considered due to the lack of the possibility of being brought into a more intensive contact with the substratum by an increased cell permeability. On the other hand, the curves representing experiments carried out with yeast cell suspensions acting either on "unphysiologic" (131) pyruvic acid, or on glucose, showed at the very beginning and later in the continued fermentation a marked excess in the production of more carbon dioxide over the unexposed controls.

The use of this α -keto acid is especially justified by two facts. When O. Neubauer (109), in 1910, discovered its fermentability, he was the first to assume that this com-

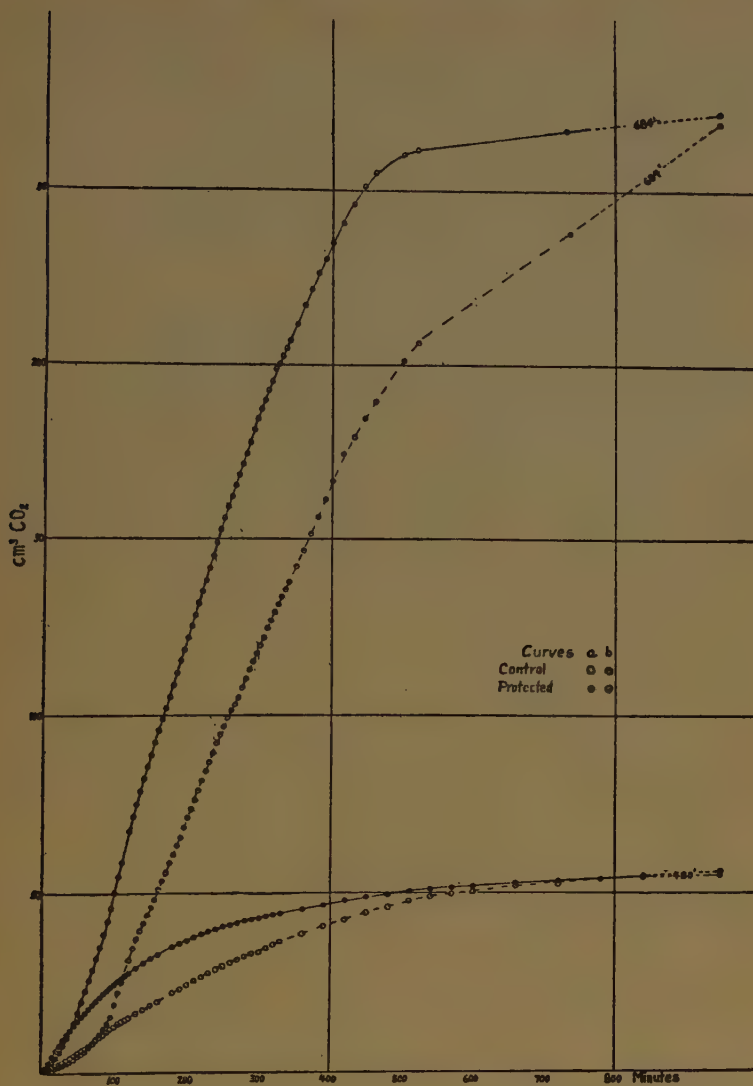


FIG. 5. (a) Fermentation of pyruvic acid, and then (b) of glucose with protected or nonprotected enzymes.

pound might be regarded as an intermediate product of the dissimilation of carbohydrates by means of zymases.

The above mentioned fact was questioned in 1911 by C. Neuberg and Hildesheimer (114), who stated:

“Aus diesen Versuchen folgt, dass die freie Brenztrauben-saeure nicht, wohl aber ihre loeslichen Alkali und Erdalkali-salze mit Hefe ‘gaeren’.”

In spite of this opinion it is now generally accepted that this acid plays an intermediate rôle in the course of fermentation, existing there presumably in the “transportation form,” to which reference has before been made (132).

Not in any instance was it observed that an irreversible decrease of permeability by means of ethylene took place, but it was found that, in agreement with the assumption made above (page 24) as to the difference in the energy content of the adsorption film, this film sometimes delayed for a short time the course of the reaction. The effect as protector always made its appearance when the effect of overcharging had disappeared.

The effect of the ethylene on living single cells or cells in tissues, such as leaves, appears to be an effect of increasing the permeability of the cells, accordingly excluding further maintenance the conclusion of Willaman (185) that anaesthetics are capable of performing enzyme synthesis *in vivo*. Simultaneously with this there also occurs a phenomenon which might be regarded as the effect of a reversible narcosis protecting at the same time the enzymes themselves from the damaging effect of the metabolism products of the yeast cells, through the formation of an adsorption film on their surface. In the case where cell-free enzyme solutions were dealt with, no influence on permeability could be observed. Assuming the correctness of these interpretations, it might be

regarded as difficult at present to regard as valid the hypothesis that the enzyme-substrate complex belongs in the group of real molecular combinations (see above, re: P. Pfeiffer), especially in so far as zymases and fermentable sugars are concerned.

In the meantime these observations offer a plausible explanation for artificial ripening processes by means of different gases. In accordance with the considerations of Nord and Franke there is no exclusion of the possibility that the idiom "activation" is absolutely meaningless in its present use (153). Since the phase of zymogenes—if its existence according to our present uncertain knowledge could be considered as justified—may be regarded doubtlessly as transcended an "activation" of the enzymes in the cases concerned does not, therefore, take place. By exposing different unripe fruits or cells of tissues to polar compounds, there appears to occur nothing less than an increase of the permeability of the cells, thus promoting the formation of the reactant-enzyme complex, and in this way advancing the hydrolysis of starch and origination of sugar and other transformation products and at the same time, through the building up of the adsorbed protector film, enabling the enzymes to act for a prolonged time under conditions closer to those of ideal cases.

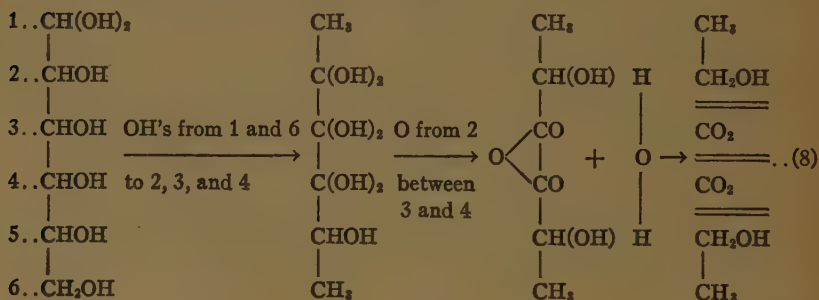
CHAPTER V

THE CONVERSION OF SUGARS INTO COMPOUNDS OF THE THREE-CARBON CHAIN SERIES

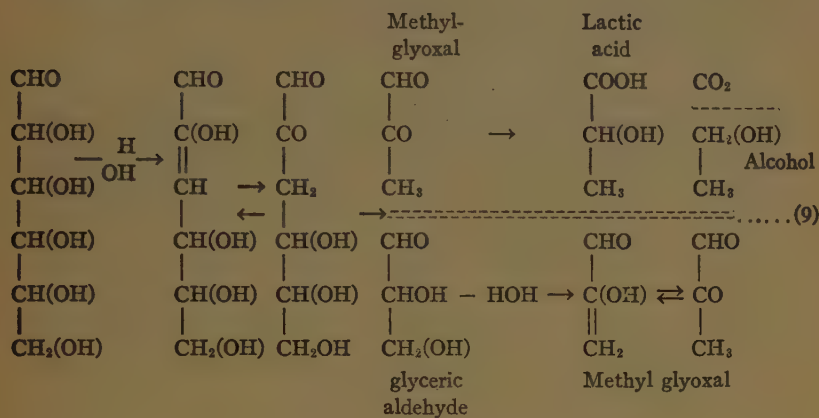
Lavoisier (88) first pointed out that during the decomposition of the sugar molecule in the course of alcoholic fermentation, the molecule not only splits into two parts to form alcohol and carbon dioxide, but he also clearly indicated that alcoholic fermentation involves the phenomena of reduction and oxidation, i.e., desmolythesis. He states:

“Les effets de la fermentation vineuse se réduisent donc à séparer en deux portions le sucre, qui est un oxyde, à *oxygéner* l’une aux dépens de l’autre pour former l’acide carbonique, à *désoxygéner* l’autre en faveur de la première pour en former une substance combustible qui est l’alcool; en sorte que, s’il était possible de recombinaer ces deux substances, l’alcool et l’acide carbonique, on reformerait du sucre.”

In 1870 v. Baeyer (7) expressed the assumption that in the course of sugar decomposition the splitting out of water in one place in the sugar molecule and the addition of the same in another plays an important rôle. If one further assumes an oxygen migration from the end to the middle of the molecule, one can derive a schematic way of showing the formation of lactic acid as well as alcohol and CO_2 :



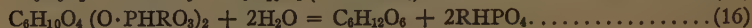
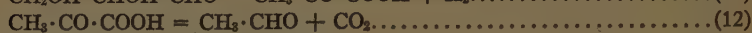
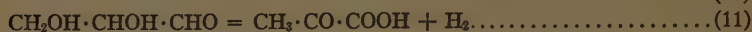
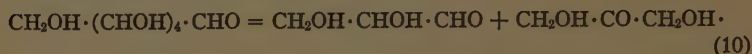
From considerations based on investigations carried out by Wohl and Österlin (189), Baeyer's assumed displacement of oxygen from the end to the middle of the chain could no longer be considered as taking place in fermentative reactions. The central idea of the scheme proposed by Wohl (96) in 1904 (in its characteristic features it is still considered valid) rested on the fact that in hydroxy compounds there can readily be a splitting out of water.



The six-carbon compound which is believed to be ready for decomposition in this scheme is the methyl glyoxal-glyceric aldehyde aldol, which would form, through an easy hydrolytic splitting, two fragments of three carbons each, viz., methyl glyoxal and glyceric aldehyde. As an illustration of the relationship it may be recalled that Pinkus (150) observed the formation of methyl glyoxal when glucose was warmed with caustic potash, and Wohl (l.c.) obtained it from glyceric aldehyde itself.

v. Lebedew and Griaznoff (92) made another deduction in which they take the stand that the hexose molecule decomposes into glyceric aldehyde and dihydroxy-acetone. In the further

course of the change, it is considered that the glyceric aldehyde forms the easily fermented pyruvic acid while the dihydroxy-acetone forms a hexose diphosphate:

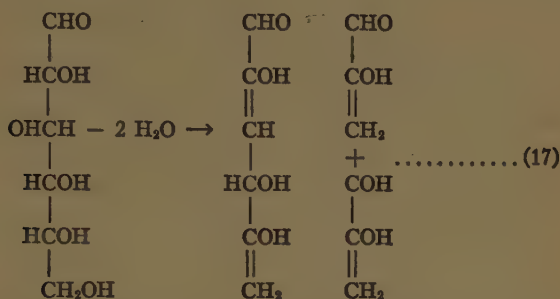


For the time, however, a real objection can be raised as to the correctness of both conceptions. One has a right to assume that if glyceric aldehyde, dihydroxyacetone, or methyl glyoxal are really the intermediate products of alcoholic fermentation, at least one of these substances must be fermentable. In spite of the work of such well known investigators as Buchner, Emmerling, Färber, H. O. L. Fischer (45), Harden, Levite, Neuberg, Schwenk, Slator, Young and others, the uncertainty concerning this question is not removed, and, lacking clear, valid proof of their fermentability, we are forced to draw on other compounds of the three carbon series to explain the intermediate steps in the fermentative decomposition of sugar.

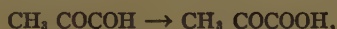
Supported by the discovery of Fernbach (42) which was later confirmed by Aubel (3), viz., that methyl glyoxal could be demonstrated in the carbohydrate splitting by bacteria, Neuberg and Kerb (115) have recommended that the hypothetical formation of methyl-glyoxal-glyceric aldehyde aldol be replaced in the Wohl scheme by that of methyl-glyoxal-aldol. The split products of the former compound have been shown, through biological means, as yet only partly to fit into the deductions of the fermentation reactions.

For avoidance of the glyceric aldehyde in the transformation

into the three carbon chain series, this change makes possible the assumption of the formation of two mols of methyl glyoxal from each mol of glucose:



If we consider once more that the acid corresponding to methyl glyoxal is pyruvic acid,



and that the relation of this acid to glucose, not only chemically but also in fermentation, is established and combine this assumption with the important observations by Battelli and Stern (8), Embden and Baldes (29), Trillat and Sauton (177), Wieland (183) and Kostytschew (85) (according to whom aldehydes can undergo fermentatively the Cannizzaro reaction), then we are in a position to find a method of explaining the formation of the end products of alcoholic fermentation.

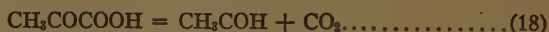
CHAPTER VI

THE INTERMEDIATE PRODUCTS

At the beginning of this century an hypothesis was expressed and an investigation reported which have influenced up to the present in undiminished strength the development of the chemistry of fermentation. The hypothesis and research developed entirely independently of each other. In the course of his fundamental researches concerning metabolism, Magnus-Levy (100) expressed on March 14, 1902, the view that acetaldehyde is a split product of carbohydrate, and on November 1, 1910, O. Neubauer (110), in connection with the comprehensive investigations by Neubauer and Fromherz concerning the decomposition of amino acids by yeast fermentation, made in the form of an unpretentious note the following communication:

“Weiter ist zu schliessen, dass die hier als Zwischenprodukt auftretende Brenztraubensäure durch gärende Hefe unter Reduktion zu Kohlensäure und Alkohol zersetzt wird, d.h. mit anderen Worten, dass sie leicht vergäerbar sein muss. Eigens angestellte Versuche, die noch nicht voellig abgeschlossen sind, haben die Richtigkeit dieses Schlusses bestaetigt. Damit ist nun ohne weiteres der Gedanke gegeben, die Brenztraubensäure koennte ein Zwischenprodukt bei der alkoholischen Gaerung des Zuckers sein.”

Later Neuberg and Karczag (116)¹ were able to prove the principal part of this communication by showing that the fermentation of pyruvic acid proceeded according to the simple equation:



¹ Compare p. 32.

This reaction is brought about by the enzyme carboxylase which occurs in all zymases and takes place without the release of free energy. It represents a typical case of the breaking down of a carbon chain by an enzyme.

The zymases necessary for carrying out this splitting are not simple ferments but enzyme complexes in which the pyruvic acid splitting ferment, the "carboxylase," occurs. The pyruvic acid, is, on the contrary, potentially represented in glucose or other fermentable carbohydrates. Through the important fact that carboxylase can break a carbon chain, this ferment assumes a special place among the known enzymes. In the fermentation of pyruvic acid, i.e., in the case of a non-sugar undergoing a characteristic change by means of yeast, we see the first case of a sugar-free yeast fermentation.

The essence of this process is that the carboxylase splits out CO_2 from α -keto acids and α -keto dicarboxylic acids, leaving behind as a residue the aldehyde of the next member lower in the series. This aldehyde is left in an unusually reactive form.

Through the circumstance that pyruvic acid not only stands in many close relationships to glucose (see page 37 and below), but also, for example, is the α -keto acid corresponding to alanine (as ketoglycerolacid to serine (133), there is some indication that carboxylase may have a predominant place among the proteolytic enzymes since we know from experiments of Neubauer and Fromherz mentioned above, that the amino-acids go over, in general, intermediately into the corresponding ketonic acids,

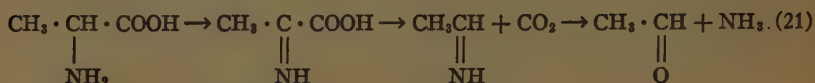


by splitting of the amino groups. The ketonic acids, in turn, by alcoholic fermentation liberate similar alcohols which, according to Ehrlich (27) are produced through the fermenta-

tion of amino-acids in the presence of a large amount of sugar by living yeast only:



It remains undecided whether in this process the cleavage of the NH_2 -group takes place first and then the α -keto acid is formed, or whether in the sense of the models of Wieland and Bergel (184) the amino group is converted to imine and later, having lost carbon-dioxide, the hydrolytic separation of the $<NH$ -group follows:



We must further consider that the transformation of pyruvic acid to lactic acid produces an increased hydrogen ion concentration (since the dissociation of pyruvic acid is 3.6×10^{-13} that of lactic acid being 1.38×10^{-4}). The fermentation of salts of pyruvic acid, on the other hand, brings about a change of the hydroxyl ion concentration, so, according to Neuberg, there can be no exclusion of the assumption that the carboxylase, through the separation of CO_2 , executes the function of regulating the reaction of the fermentation medium.

The connecting rôle of α -keto acids, which represent common ground between proteins and sugar, manifests itself also in the phenomenon that these acids are supposed to serve as "stimulators" in alcoholic fermentation. This is believed to have been proved also by experiments which aroused the impression that the co-enzyme of yeast, in the presence of potassium phosphate, can be replaced by a mixture of different α -keto acids such as occur in yeast protein. Meyerhof was not able to confirm this statement using pyruvic acid (compare also v. Szent-Györgyi, l.c.).

The fermentability of the "unphysiologic" pyruvic acid or the properties of the above mentioned decomposition products constitutes full confirmation of Neubauer's assumption and experiments that this acid is an intermediate product in alcoholic fermentation, or a split product of the sugar molecule; for, in contrast to its ability to withstand the action of a temperature up to 165° and of concentrated sulfuric acid up to 150° , in the presence of a great excess of various yeasts, it is, in a more or less short time, split (70 to 80 per cent) into CO_2 and acetaldehyde.

Apart from the biological and earlier (page 00) discussed chemical connections, there is also a hint of genetic connection to the glucose in that it may be formed endothermically by the oxidation of lactic acid,



or by the dehydration of glyceric acid:

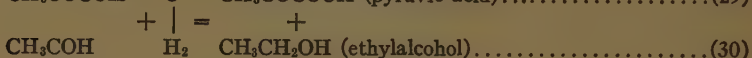
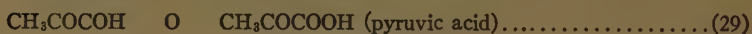
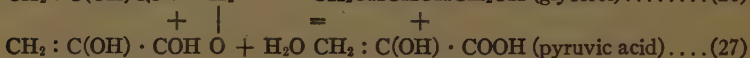
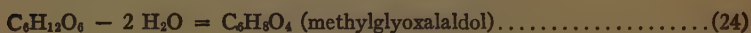


In the above given scheme of decomposition we became acquainted with the compounds in which carbonyl and ethylidene radicals are represented, so that their origin is withdrawn from "paper chemistry" discussions and is placed within the range of experimental proof.

The connection between the assumption of Magnus-Levy and the discovery by Neubauer is therefore clear at first sight. In the case of the fermentation process it can be established that proof of the origin of CO_2 is given. Biologically speaking, it is the same whether acetaldehyde and CO_2 occur as such or are held fast in the form of their combination, i.e., pyruvic acid, all the more as the heat of combustion of pyruvic acid, recently determined by Blaschko (10), is identical with that of acetaldehyde, indicating that decarboxylation occurs without energy transfer (compare chapter VII).

The explanation of the formation of alcohol is not as simple. Battelli and Stern (l.c.), and Parnas (146), have demonstrated the occurrence in animal tissues of a ferment which acts upon two mols of an aldehyde taking up water to give a Cannizzaro reaction. That is, the aldehyde is rearranged to equal molecular amounts of alcohol and acid. An analogous observation was later also made by Kostytschew (l.c.) in the case of the action of yeast on acetaldehyde. But a Cannizzaro reaction on the acetaldehyde formed in the decomposition of pyruvic acid gives ethyl alcohol itself.

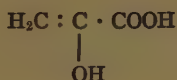
By means of a suitable arrangement of the above mentioned discoveries, and accepting the sugar from the decomposition of the hexose phosphates to be in the "transportation from" (see page 15) the earlier discussed scheme of Wohl (see page 35) may be completed in the following way:



Omitting processes at present not experimentally clear (processes which are concerned in the formation of the three-carbon chain series), methylglyoxal is considered the first product having three carbon atoms in the molecule. It may be formed after the splitting out of two molecules of water from the hexose molecule, which perhaps requires the intermediary existence of methylglyoxal aldol. All further processes then are brought about through repeated Cannizzaro rearrange-

ments on methylglyoxal. In the case of the pyruvic acid first formed, the addition of water gives glycerine. If we assume the isochronous formation of acetaldehyde and CO_2 by the action of carboxylase on the pyruvic acid, then a Cannizzaro reaction must take place between two *different* aldehydes. The result of this phenomenon is the formation of ethyl alcohol on the one hand and the re-formation of pyruvic acid on the other. From the latter the carboxylase always produces new CO_2 and acetaldehyde. We see, therefore, an uninterrupted formation and decomposition of pyruvic acid, which, just as methylglyoxal, can not then be accumulated. After this restless conversion naturally some acetaldehyde would be left over. This assumption is in harmony with the fact that in fermentations traces of acetaldehyde are always detectable, equivalent to some 0.15 per cent of the raw materials used.

One has the impression that the above mentioned facts may also be supported by the important findings of Henri and Fromageot (47, 65). These investigators have shown, by measuring the absorption spectra of pyruvic acid, that the quantitative relations of the keto and enol forms of this acid, under conditions of concentration that approach closely to those of biological processes, are strongly dependent upon the hydrogen ion concentration. The much more reactive enol-form



could thus also be responsible for the fermentability of the acid in normal fermentation media.

However, when one considers the above statements in connection with the repeated assertion that the velocity of fermentation of pyruvic acid at ordinary temperature can several times exceed that of glucose, which has been deduced from

experiments carried out mainly in the presence of salts of sulphurous acid in which the hydrogen ion concentration was not measured, one will not be able to rid oneself of a certain feeling of uncertainty as to the actual rôle of pyruvic acid in these processes.

Fortunately, it does not seem necessary to question (34) this proposition when we take into consideration that the experiments of Neubauer or of Neuberg, Hildesheimer and Karczag are not interdependent from a biological standpoint. In contradiction to this, the assertion that pyruvic acid is in the absence of added buffers subject to faster fermentation than sugar could not be confirmed by the precise investigations of Lebedew (89) (1917, 1924), Hägglund and Augustson (1925) and others, all the more as the experiments controlling (1911) the findings of Neubauer on the fermentability of the pyruvic acid were carried out under unphysiological conditions. The above mentioned measurements of the absorption spectra by Henri and Fromageot show that under conditions of biochemically permissible concentrations, the acid is only present in the readily fermentable enol form. On the other hand, we know that the pyruvic acid is a very strong acid ($K = 0.56$) and since it is so highly dissociated, in accordance with the observations of Brenner (12), Brooks (13), and others, it may only enter uninjured cells or reach the place of enzymatic activity with great difficulty if at all. The connection of these observations is clear! The acid which is originated in a biochemical process (that is to say, within the cell), is present in the readily fermentable enol form. It will be isochronously decarboxylated with the same speed as the transportation form of the sugar is formed. In contradistinction to this, when pyruvic acid is added to the mash itself, it is in an uncomparable degree more highly concentrated and will be fermented only in such proportion as the

enol form is present and ready to undergo disintegration. This again is dependent on its ability to penetrate into the cell. We see, therefore, in conformity with earlier results, the outstanding significance of the transportation form² of a compound which is indispensable to the initiation of a biochemical reaction.³ The same importance also attaches to the "isochronic rearrangement" of this form, to which reference has recently been made (134) in certain connections. It may, therefore, be regarded as certain that "unphysiologic" pyruvic acid is fermented slower than sugar in contra-distinction to the "biologic" acid which ferments practically with the same speed as sugar. It appears equally probable that considerations of structural organic chemistry alone are hardly suitable as justification of positive or negative conclusions drawn from the macrochemical behavior of methylglyoxal, hydroxypyruvic aldehyde or related compounds under biological conditions. Accordingly in intracellular reactions there does not appear to be any logical basis for the calculation of a quotient based upon the rate of the fermentation of glucose as compared to that of pyruvic acid.⁴

Before entering into discussion of the evidences that form the experimental basis of this scheme (see section 7),

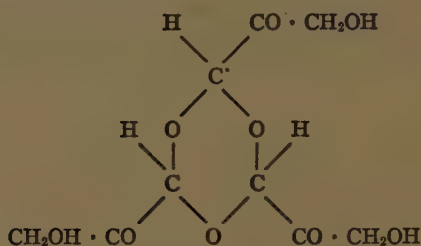
² There probably also belong in this small group some sulfur containing compounds, newly described and investigated, which were supposed to have a decisive rôle in reversible physiological processes, and certain compounds of the bile promoting the hydrodiffusion of the cell (78).

³ It does not seem desirable at present to complicate the conception by analyzing the influence that the transient acid must have on the interfacial tension of the membrane.

⁴ In view of these considerations it is easy to understand that R. Nilsson and E. Sandberg (126) feel clearly the uncertainty of their conclusions. Otherwise they would not have been weakened by the authors themselves through the formulation: ". . . . also noch nicht ganz überzeugt. . . . "

attention might be drawn to the instances in which further difficulties are brought to light by critical consideration.

It is quite true that the fermentability of methylglyoxal by yeast has not as yet been demonstrated (4). This circumstance nevertheless does not conclusively eliminate the assumption that the compound can be an intermediate one in alcoholic fermentation, since, according to results⁵ obtained through Miss Mollie G. White, that *Fusarium lini* B. may utilize the readily accessible hydroxypyruvic aldehyde (39):



as a sole source of carbon. This powerful fungus (70) possessing according to Anderson (2) and to Tochinai (174) an astonishingly wide optimum range of pH (3.5–9.5), when grown on glucose shows a metabolism which is in essential agreement with the metabolism of yeast. Further proof of the fact that *F. lini* grows well in nutrient liquids in which the sole carbon source is acetaldehyde, acetone or dihydroxyacetone, has been given by the use of a fermentation tube (142) shown in the drawing (Fig. 6). The much weaker growth in nutrient media containing pyruvic acid, may be considered as further evidence of the viewpoint that “unphysiologic” pyruvic acid undergoes fermentation more slowly than, or acts differently to the “biologic” acid. The development of pigment observed in these experiments was in good harmony with the findings of Sideris

⁵ Compare the footnote added at correction in *Protoplasma* 2, 303 (1927).

(163). The results intimated here represent the first experimental proof of the conformity of the intermediate processes in the case of yeast and *Fusarium lini* B. This is of great importance since the dimensions of this fungus must readily allow determination of the hydrogen ion concentration of its protoplasm, thus rendering possible the drawing of *per analogiam* conclusions from the relation of this H ion concentration to that found within the yeast cells and to eliminate



FIG. 6

the uncertainties caused by the results of the very original method of Tait and Fletcher (155, 172).

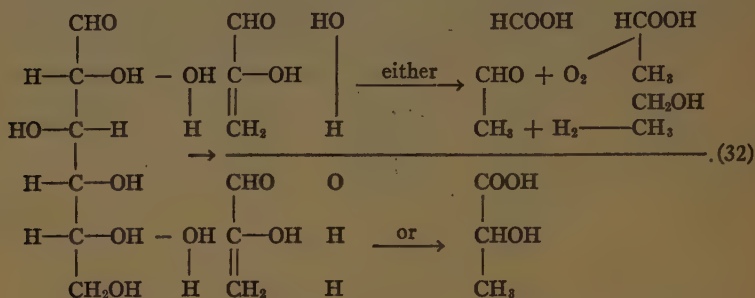
It should be mentioned that Dakin and Dudley (20), as well as Neuberg (112), state that they have found in yeast, muscle and other animal organs, an enzyme which brings about an internal Cannizzaro reaction:



thereby forming lactic acid. This acid is well known to appear in the products of fermentation of sugar by yeast juice. These

results were recently confirmed by Kuhn and Heckscher (87), but they were unable to demonstrate any reversibility in the course of the reaction.

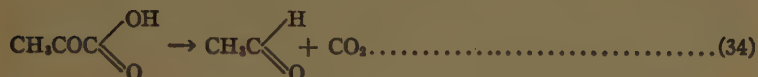
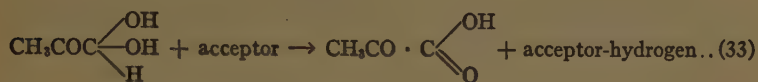
An interesting alternative hypothesis to the Magnus-Levy-Neubauer theory was formulated recently by Lusk (99) to explain the carbohydrate katabolism in muscle:



Under anaerobic conditions the methylglyoxal originated from glucose is converted into lactic acid, and under aerobic conditions it is changed to acetaldehyde and formic acid. There are available as yet only indirect evidences in support of this scheme. However, it accounts not only for the presence of acetaldehyde where carbohydrate is dissimilated, but also for the occurrence of formic acid. Therefore, there are both chemical (see page 00) and biological grounds which give warrant for assuming the intermediate appearance of methylglyoxal.

In some recent communications worthy of note but not yet adequately supported by experiment, Kluyver and Donker (81) have turned against the idea of a mixed Cannizzaro reaction. The essential thing in their hypothesis in which they attempt to apply Wieland's theory of dehydrogenation in processes of aerobic respiration to anaerobic dissimilation, is that all parts in the process of sugar decomposition are none other

than coupled dehydrogenation-hydrogenation reactions. Correspondingly, alcohol would be formed from a dehydrogenation of methylglyoxal hydrate through passage of the hydrogen to the acetaldehyde.⁶



They state:

"On the basis of these facts it is evident that there exists no reason whatever to have recourse in certain cases to the narrower explanation offered by a Cannizzaro transformation."

We shall see later that these explanations are automatically weakened by experiment (chapter VII, paragraph B).

⁶ Compare also A. Lebedew, Bull. soc. chim. France, [4] 11, 1040 (1912).

CHAPTER VII

CONCLUSIONS

Dumas (24) showed many years ago that alcoholic fermentation can proceed in the presence of various alkali salts, and in 1874 he correctly hinted the possibility of influencing alcoholic sugar decomposition by the presence of alkali sulfites. Later Mueller-Thurgau and Osterwalder (106) observed that in the case of fruit juices or fermenting sugar solutions, added sulfurous acid instantly combined with the correctly assumed acetaldehyde present. It was obvious that this compound might be the acetaldehyde-sulfurous acid of Ripper (156) (1892), the sodium salt of which, known since the time of Bunte (16) (1873), is so important for our following discussion.

In the course of extensive investigations concerning the hydrolytic splitting of bound sulfurous acid, Kerp and Laudon (79) have established that the dissociation constant of acetaldehyde sodium bisulfite (2.84×10^{-6}) to the corresponding glucose compound (311×10^{-8}) averages about 1:90,000.

Proceeding from the knowledge of the above mentioned facts and from the desire to increase as much as possible the yield of glycerol in alcoholic fermentation (see page 42), Connstein and Luedecke (19) began pertinent experiments in 1914. The ordinary fermentation of sugar always takes place either in neutral or slightly acid solution. Should, however, the possibility exist of removing the acetaldehyde (equation 28) formed as Bunte's compound, it is to be expected that in the case of an appropriate method of fermentation amounts of glycerol could be obtained which are not inconsiderable.

The first investigations were made with various alkaline substances such as disodium phosphate, sodium acetate, sodium

bicarbonate, ammonium carbonate, etc. However, the alkaline mash brought to light a disagreeable characteristic in a short time, viz., they formed an excellent nutrient medium for all possible acid forming bacteria, especially lactic strains. These bacteria consumed not only the greater part of the sugar, but also so contaminated the glycerine that it was very difficult to purify. Investigations of the manufacture on a large scale with disodium sulfite were then begun. The yeast tolerated this salt in increasing quantities, which, when added in large amounts to goods to be fermented, exerted a pronounced antiseptic action.

A. With just a hint as to the complete interpretation found elsewhere (135), mention of technical investigations in this case has significance. The rather infrequent occurrence where practical workers have very fruitfully and stimulatingly influenced theoretical research in this sphere is well manifested here. Chemists in the United States (Eoff, Lindner and Beyer (33)),¹ Germany, England (Cocking and Lilly (18)), the former Austria-Hungary, Switzerland (Schweizer (161)), and Japan (Tomoda (175)), vied with each other in the endeavor to solve the problem, and it remains to the merit of Faerber, Hirsch, Neuberg, Reinfurth and Ursum (68) on the one side and Reik, Pollak, and Zerner (154), on the other, to have produced a significant analytical insight and a part of the proof for the scheme given on page 42.

¹ The task of investigating this problem was assigned to these workers under date of May 9, 1917, after Dr. Alonzo Taylor, at that time Assistant Secretary of Agriculture, had been in Germany the preceding summer. He learned there, presumably in 1916 "That the Germans were producing glycerine in large quantities by a fermentation process, sugar being the material used." Under these conditions it is difficult to understand, how certain investigators could claim in 1919 and continue to do so that their work was originated "independently" of the findings of Connstein and Lüdecke patented in 1915.

Starting out from the observation of Fernbach and Schoen (43), that pyruvic acid can be isolated during the course of alcoholic sugar decomposition, which was long opposed by Neuberg through Kerb (77) it could be proven that if one

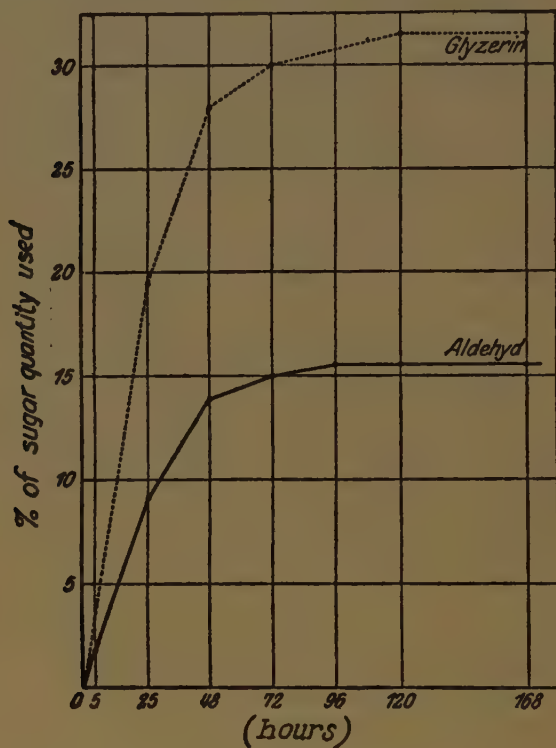
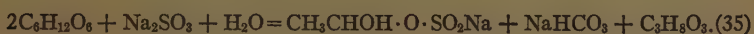


FIG. 7

carries out the fermentation in the presence of the weakly alkaline sulfites, the theoretically possible amounts of acetaldehyde and glycerine are obtained. The already mentioned aldehyde-bisulfite addition compound, $\text{CH}_3 \cdot \text{CHOH} \cdot \text{O} \cdot \text{SO}_2\text{Na}$,

which can be easily split (176) under re-formation of acetaldehyde by means of a hot soda solution, concentrates itself in the mash and can be separated. The corresponding hexose compound in the presence of water is practically completely dissociated. Compared with ethyl alcohol, acetaldehyde is considered as an oxidation product, and hence the presence of a reducing compound is to be expected. This assumption really proved to be correct, since the hydrogen atoms, which can not act upon the acetaldehyde because of its formation of a compound with Na_2SO_3 , make possible the formation of glycerol by acting upon another half molecule of sugar:



Detached from the binding to the alkali salt, the equation takes the form:

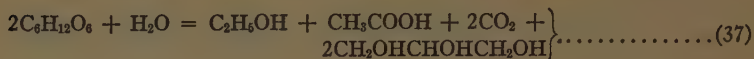


and according to the curve in figure 7 (Biochem. Z., 98, 153) the ratio between glycerine and acetaldehyde at any given time is constant.

It is quite worthy of note that the blocking of the acetaldehyde can be brought about not only in a chemical way but also, as Abderhalden, Glaubach and Stix (1) showed, through adsorption. They have proven that the above reactions can be made to proceed in an approximate manner in the presence of animal charcoal.

If the fermentation be carried out in the presence of simple alkali salts, which, in contrast to the bisulfites, have no specific action on the acetaldehyde formed, only traces of the latter can be found. It undergoes a Cannizzaro transformation and can be found again also in molecular proportion to

glycerol, but as acetic acid and ethyl alcohol. The proportions can be expressed in the following equation:



and show that for every mol of acetic acid there are always two mols of glycerol.

On the basis of the above facts, v. Grab (53), working with C. Neuberg, confirmed the statements of Fernbach and Schoen by carrying out a Doebner synthesis of α -methyl- β -naphtho-cinchonic acid in a cell-free fermentation of sugar. This was done by the reaction of β -naphthylamine with pyruvic acid:



On the other hand Aubel and Salabartan (5) showed that pyruvic acid was an intermediate product in the coli fermentation of glucose (see below). When we take all these things into consideration this part of the discussion may be considered closed although Lebedew (90) has expressed doubt concerning the data produced by v. Grab.

B. After the above discussion (without mention of equations 24 and 25 on page 42) only the correctness of the assumption of a Cannizzaro reaction between two *different* aldehydes remains in doubt. Several investigators have pointed out the necessity of proof to strengthen the assumptions involved.

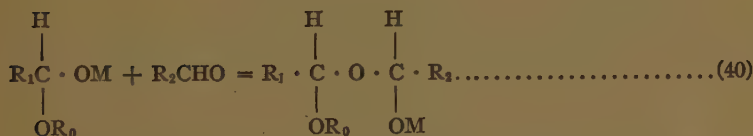
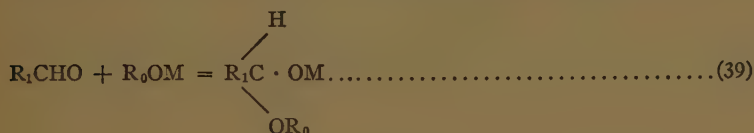
During the last years extensive investigations to demonstrate this reaction have been taken up by Endoh (31), Nakai (107), Nord (136), and others, and in 1921 v. Grab, stating (l.c., page 71):

“ durch den von Nord erbrachten Nachweis, dass in der Tat eine gemischte Dismutation zwischen ungleichen Aldehyden der aliphatischen Reihe moeglich ist,”

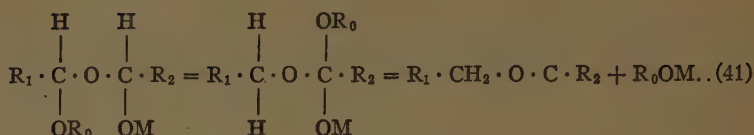
was able to rely upon the feasibility of the assumption of a mixed Cannizzaro reaction as founded on certain principles. All together the preliminary work of the Lieben school did not clear up the question.

Meanwhile these catalytically influenced model investigations have been completed, and we now know the following examples: acetaldehyde plus isovaleric aldehyde, isovaleric aldehyde plus benzaldehyde, acetaldehyde plus benzaldehyde, acetaldehyde plus furfural, furfural plus isobutyric aldehyde, acetaldehyde plus chloral, acetaldehyde plus bromal, acetaldehyde plus cinnamic aldehyde. Indeed, the reaction can take place between most widely differing kinds of aldehydes, and afterward the original suggestion was made by Nord (1924) the indications of Verley (178) confirmed the experiments according to which this reaction might also occur with mixtures of aldehydes and ketones.²

Simultaneously, the investigations have brought two other conclusions to maturity. (a) Through the formulation:



² Compare also the German Patent No. 434728/120 (1924). The mixed Cannizzaro reaction between an aldehyde and a ketone is for the first time suggested and described here.—The claim for priority of C. Neuberg and G. Gorr (Biochem. Zeitschrift, 166, 444; 1925) is erroneous. See also S. M. Gordon, J. biolog. Chem., 75, 164; 1927.



which is based upon the work of Wieland or Lachmann, it is probable that an *isochronic* rearrangement of dialkyl ethers precedes the formation of mixed esters. (b) By the application of the method to single aldehydes, several alcohols of importance in physiological investigations (32) and medical application³ have readily been made accessible.

³ Compare Industrial and Engineering Chemistry, News Edition, 5, No. 13, p. 7 (1927). "E 107" is tribromoethyl-alcohol (Avertin).

CHAPTER VIII

REDUCTIONS AND SYNTHESIS IN THE COURSE OF SUGAR DISSIMILATION

A. Of the biochemical processes found in nature, the reduction processes are of greatest interest because they are the most difficult to interpret. We see that, beside the deoxidation of CO_2 in assimilation processes, plants bring about the greatest hydrogenation performance in the synthesis of protein from sugar and ammonia. One has an object worthy of study in the reductions done by yeast cells. Since these reductions, because of lack of disposable hydrogen can be brought about only in roundabout ways—one often thinks of so-called coupled or induced reactions—the question is often asked, from where does the energy for the reduction processes come? A whole series of biological experiences assign a connection between reductions and the oxidative decomposition of carbohydrates, and especially the processes of fermentation which have been discussed appear as an alternate oxidation and reduction of the sugar molecule (Lavoisier l.c.).

In continuation of the basic observation of Lintner and v. Liebig (94) concerning the reduction of furfural by yeasts in alcoholic fermentation, the reductive action of yeast on other aldehydes was made the object of further work and it has been shown that this characteristic is so marked that the yeast could even act upon substances foreign to their organism. Experiments of "phytochemical" reductions made with various nitro compounds showed the formation of the amino compounds by way of the corresponding intermediate compounds. In the case of o-nitro-benzaldehyde (137) also, for example, the

yeast acts selectively. This is in harmony with the experiences of Nord (138, 141) on progressive catalytic hydrogenation.¹

Several years ago it was presented in a lecture that fermenting yeasts can also reduce ketones (40, 120, 162). The reaction takes place as with methylheptenone in both the aliphatic and aromatic series, and as it is an assymetric one, it furnishes a method of obtaining optically active secondary alcohols otherwise difficult to prepare. Diketones (121) undergo the same change and give glycols. Examples of this class which have been studied are diacetyl and benzil. It has been definitely proven that these reductions are not purely chemical in nature, but are catalyzed by ferments. Thus if one uses a racemic aldehyde, e.g., racemic valeric aldehyde, one gets optically active amylalcohol, or, beginning with diacetyl, one gets optically active butylene glycol.

The alcohols in nature result from the corresponding carbonyl compounds, i.e., from aldehydes and ketones. Regarding the thioalcohols (the mercaptans), the possibility of their origin from the thioaldehydes could also be substantiated. For this purpose one uses in the phytochemical reduction the ammonia derivatives of the thioaldehydes (the thialdines (122)). In this case it must be evident that the origin of these compounds in nature appears entirely possible, since ammonium salts are available everywhere. In the case of ethyl mercaptan, however, it can be shown that the formation of the mercaptan is purely an enzymatic process, as it can be brought about in cell-free fermentation by the use of yeast juice.

¹ It was the first time here that evidence was furnished for the occurrence of an intermediate stage in the course of a catalytic hydrogenisation. By this "trap method" the passing of the hydroxylamin stage was experimentally proven.—It is worthy of note that Blom (Biochem. Z., 194, 392; 1927) recently succeeded in the demonstration of hydroxylamine as an intermediate product in the course of nitrate assimilation through certain soil bacteria by using acetone as a "trap" agent.

In the course of these investigations acceptance of the idea that the reduction of various compounds has a relation to the simultaneous course of the processes of alcoholic sugar decomposition is indicated, pointing to the possibility that in ultimate analysis the "hydrogen" used in reductions is fermentation "hydrogen," which, naturally, is not evolved in the free state and therefore requires an acceptor.²

Since the course of sugar decomposition seems to involve pyruvic acid and acetaldehyde as intermediate steps, and since the former is an oxidation product of half a sugar molecule, it is necessary, if there is to be any change in this oxidation product, for an equivalent reduction process to occur simultaneously.

Normally the necessary acceptor is formed through the further splitting of the pyruvic acid, occurring in the form of acetaldehyde which takes up the "hydrogen" and passes over to alcohol. If this representation is correct, other hydrogen acceptors, i.e., phytochemically reducible substances, must work in an analogous manner to acetaldehyde.

But the fact of the reduction of many compounds does not in itself say anything concerning the course of the process. The yield of the reduction product only emphasizes that no process like a Cannizzaro rearrangement can come into play, not mentioning that simple ketones and nitro compounds are not capable in general of a transmutation. We are inclined then to devote some consideration to the fact that it has been possible to demonstrate acetaldehyde in such biochemical fermentations. In singular cases the acetaldehyde has been found in amounts almost equivalent to the reduction product. One is led to believe that here indeed the added reducible compound has become the acceptor for the fermentation hydrogen instead of the acetaldehyde and, in agreement with that,

² Compare in the case of sulfur the interesting statement of A. Hottinger (Schweiz. med. Wchnschr., 53, 430, (1923)).

has pushed aside out of the reaction an equivalent amount of acetaldehyde.

It is interesting to note in this connection the fine demonstration by Kluyver and Donker (82) of the origin of acetyl-methyl carbinol and 2,3-butylene glycol, corresponding to the earlier results of Harden, Walpole, and Norris (60), in the fermentation of glucose in the presence of methylene-blue or sulfur. The products of decomposition occurring in the fermentation of fructose also have, in this sense, the effect of a hydrogen acceptor.

If, accepting the view of these authors, we include oxygen in this sphere, the aforementioned oxidation phenomena would in addition to this take on a very original and attractive interpretation (see page 2).

We must remember however that yeast co-ferment can be simulated in its action by a mixture of various α -keto acids (see page 39) (but not by insulin (160)). According to Kendall (76), thyroxin acts as a catalyst, receiving its action by being alternately oxidized and reduced; that is, it acts simultaneously as donator and acceptor.³ Should it be found that thyroxin and the bios isolated from autolized yeast by Eddy, Kerr and Williams (25), are joined in relationship on the basis suggested by Kendall, then perhaps further investigations may place us in a position to seek an explanation of the reductive processes in yeasts in this direction.⁴

B. Reference has already been made (see page 39) to the fact that zymases are not uniform, but constitute enzyme complexes in which various tasks fall to the different parts. As

³ Compare also F. Knoop and H. Oesterlin, *Z. physiol. Chem.*, **148**, 301, 302, (1925).

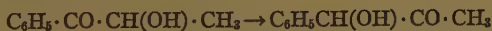
⁴ In the meantime the former formula of thyroxin and (according to a private communication from Dr. Edward C. Kendall) the analysis of bios have been found to be erroneous.

ways of checking up this opinion increase in number, it is interesting to note the facts that can be cited as confirmation of this assumption. In the fundamental investigations of Lintner and v. Liebig (95) the observation was made that a considerable part of the furfural added to a fermenting sugar solution was not reduced in the way described above, but apparently underwent a condensation by the acetaldehyde which appeared as an intermediary. In continued investigations they showed that the by-product was in reality the result of a direct carbon chain linkage, C-C, and, since the compound called by them furyltrimethylene-glycol, $C_4H_3O \cdot CHOH \cdot CH_2CH_2OH$, was found to be optically active, there can be no doubt that it was the result of fermentative action. In contrast with the ordinary process of biochemical katabolism of molecules, which we regard as the function of fermentation, we see herein established the first case of a carbon chain synthesis, or the carboligation action of a ferment, hitherto recognized only in its performances.

When Neuberg and Hirsch (69) continued the experiments described above, replacing furfural with benzaldehyde, optically active phenyl acetyl carbinol, (α -Phenyl-pyruvic alcohol) $C_6H_5C^*HOH \cdot COCH_3$ was obtained and, according to Hirsch (67), in fermenting pyruvic acid alone there is formed in addition to the nascent acetaldehyde methylacetyl carbinol (Acetoin):



It is quite remarkable that Methylbenzoylcarbinol is capable to undergo a rearrangement and furnishes under similar conditions phenylacetylcarbinol



* indicates an asymmetric C-Atom.

This was found by Faworsky (41) in conformity with his experiments on the rearrangement of α -ketoalcohols in vitro and intimates a different mode of origin. All the above mentioned reactions indicate, however, at present that they can take place only with nascent acetaldehyde. Neuberg and v. May (118) have also shown this by fixing the acetaldehyde with sulfite, whereby they secured a total suppression of the carboligation action. But the process of carboligation itself is not connected with the aldol reaction. It is indicated in comparison with the Benzoin condensation as "Acyloinreaction."

There occurs under raising of the potential, therefore, the conversion of an oxidative splitting process into a fermentative resynthesis of carbohydrate, for in all these cases of transformations sugar or pyruvic acid occurs which is being decomposed to give the necessary acetaldehyde for the carboligation effect.

Also, the view of Haehn (55) that acetaldehyde forms also a fundamental ingredient of fats is confirmed through the origin of higher fatty acids, when the organism, *endomyces vernalis* is used. In opposition to Smedley MacLean and Hoffert (165), Haehn and Kintoff have proven that the building up of the fatty acid molecule is accomplished in two steps, the first by the decomposition of glucose to acetaldehyde through the action of the zymases, the second by the action of the synthesizing enzymes on this aldehyde. It is impossible to state whether the pyruvic acid or lactic acid precedes acetaldehyde. The above mentioned fungus has shown itself able to assimilate both acids as well as the aldehyde.

CHAPTER IX

TRANSFORMATIONS THROUGH OTHER MICROÖRGANISMS

Not only in the case of alcoholic fermentation does acetaldehyde play an important rôle (by being one step in alcohol production) but also in cases of important bacterial fermentations in nature the same is equally true. In order to demonstrate this, the bacterial process concerned is permitted to proceed in the presence of a substance which will fix the expected aldehyde. For this purpose it has been shown that, in the case of bacterial fermentations, the neutral sulfites, such as Na_2SO_3 and CaSO_3 , can be used. They, in contrast with most of the bisulfites required for the fixation of aldehydes, cause little injury to the enzymatic and life processes, or in any case, do not stop them. The whole difficulty of this problem consists in this, that the expected intermediate products which are formed during the dissimilation of sugar to its split products, acetaldehyde and alcohol, all show labile characteristics, and, collectively, must contain the carbonyl ($-\text{CO}-$) group. Because of this fact, the choice of appropriate means of intercepting the reaction is extraordinarily limited. The compounds used under the conditions of the experiment must combine with the sugar to form a substance not readily acted upon biologically or else give no condensation product at all. This obstacle blocked all of the earlier steps attempted in this direction. The way out of this difficulty consists in selecting a compound which will intercept the reaction, one which has a fine gradation in its affinity for sugar and its different transformation products so that the least affinity is manifested for the initial material and the maximum for one of the later products of this change. (Compare Kerp and Laudon, page 49.)

Such substances, as mentioned above, are the normal sulfites. They give with the sugar an addition product in which the components are exceedingly loosely bound, being highly dissociated in aqueous solution. On the other hand the stability of the sulfite complex increases down to acetaldehyde. As normal sulfites (M_2SO_3) are not poisonous for microorganisms, it is possible to carry out fermentation in their presence. This is in contrast to free sulfurous acid (H_2SO_3) and bisulfites ($MHSO_3$) which are used in different industries based on fermentation because of their disinfecting qualities.

By this method Neuberg and Nord (123) were able to show that acetaldehyde occurred as an intermediate product in the fermentation of sugar, mannite, and glycerol, using the widespread kinds of coli and various pathogenic microorganisms.

The exchange of carbohydrates in nature by means of these agents is very great in scope. Their action extends not only to the true sugars but also to related substances as mannite and glycerol. The yeasts are relatively strongly constituted organisms which will stand the addition of a substance which takes up some compound formed in the course of the reaction. In the case of bacteria it is necessary to make a modification, using an alkali-earth sulfite (as $CaSO_3$) which gives a neutral reaction, instead of the alkali metal sulfites which have a basic reaction. The former not only have the advantage of being neutral in reaction, but also are relatively insoluble in water. Because of that, one can diminish detrimental osmotic processes in the case of sensitive microorganisms. On the other hand, one obtains only a more limited sulfite-ion concentration. This might be adjusted to a certain degree. The presence of a sulfite is the only essential.

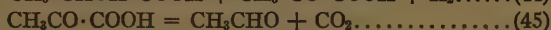
The aldehyde is clearly produced at the expense of the reaction which normally leads to the production of ethyl alcohol or

acetic acid. Harden's (61) investigation of the coli fermentation gave as the normal reaction:



In the presence of sulfite, aldehyde was obtained in the appreciable amount of 40 to 45 per cent of the quantity of alcohol concerned.

The aforementioned authors believe however that the acetic acid and the alcohol are derived by a Cannizzaro reaction from acetaldehyde. If this is true the equation given above for the breaking up of the glucose by colon bacilli may be interpreted to mean that pyruvic acid and acetaldehyde come next. This gives the following equations:



The similarity which has often been noted between fermentation of yeast and colon bacilli would be based on the fact that in the latter, also, that part of the sugar which does not change into lactic acid is decomposed by way of pyruvic acid and acetaldehyde. The reduction of acetaldehyde in the last phase does not take place in fermentation by colon bacilli because the hydrogenizing hydrogen is developed freely in the molecular state, while at the same time the acetaldehyde primarily produced becomes a simple transformation into alcohol and acetic acid.

The recent results of de Graaff and Le Fèvre (52) seem to coincide with this viewpoint. According to them the methyl glyoxal is not decomposed by the bacteria of the colon-typhoid bacillus group by way of acetaldehyde. However, they found acetoin. Similar results were obtained lately by Kaziro (75) who has proven that *B. subtilis* is able to produce acetoin from

glycerol under certain conditions. However it is not yet decided, whether any rôle is to be ascribed in this process to the added desoxybilianic acid. Neuberg and Gorr (119) noted the effect of lactic acid bacteria (*B. coli*) on methylglyoxal and found that this unstable compound was converted to lactic acid, just as Aubel and Salabartan (l.c.) had noted in their observations. It has to be mentioned, on the other hand, that according to Virtanen, Karström and Bäck (180) certain lactic bacteria are unable to act on methylglyoxal, glyceric aldehyde or dihydroxyacetone. In distinction to this the recent findings concerning the anaërobic fermentation of glucose by *B. coli communis* are entirely contradictory. Grey and Young (54) have shown that even under these conditions there occurs a considerable production of gas; whereas Róna and Nicolai (158) claim to have observed the quantitative splitting into two molecules of "fixed acid," obviously lactic acid.

Aubel and Genevois (6) have the opinion that the hydrogen developed in biological processes of this kind is incapable to exert reduction effects. This is not yet certain but could be well tested by performing experiments of phytochemical transformations (see page 57) in the presence of these microorganisms.

The sodium salts of hexosemonophosphoric and hexosediphosphoric acids were also decomposed by *B. coli communis* in atmospheres of oxygen or nitrogen. The products were alcohol, formic-, lactic-, acetic- and succinic acids. This result of Manning (102) is quite interesting in view of the unsettled question of the unfermentability of the diphosphates by living yeasts.

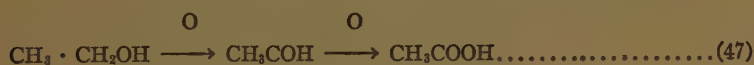
Similar information as that obtained with the colon bacteria was also obtained for the metabolic processes of two pathogenic microorganisms, viz., the dysentery bacillus and the gas gan-

grene bacteria. The known kinds of dysentery bacilli, the Flexner, Y, and Shiga-Kruse, all give acetaldehyde in cultivation in solutions of maltose, glycerol, and mannite. The causal organism of gas gangrene, which in the late world war was so feared in wound infection, is likewise a powerful carbohydrate consumer. This anaërobic organism related to the butyric acid bacteria grows in sugar solutions with the production of acetaldehyde. Since acetaldehyde is a violent poison for higher organisms, its occurrence amongst the products of metabolism of pathogenic microörganisms may also be of interest for the study of infectious diseases.

It can be shown that acetaldehyde is an intermediate product also in the case of butyric fermentation by *B. butyricus* (Fitz) when the fermentation is carried out in the presence of Na_2SO_3 (113). In place of butyl alcohol or butyric acid there is formed ethyl alcohol and acetic acid in connection with the splitting processes, which, beside the gaseous decomposition products of sugar, could only lead to acetaldehyde production.

The results of this investigation appear to be, however, doubtful in so far as the workers were using two kinds of bacteria (23). One stem was an obligatory anaërobe but the other was not a typical *B. amylobacter* A.M.

It seems remarkable that the fermentation of alcohol to acetic acid (124), which is technically used for acetic acid production, also passes through the acetaldehyde stage:



If the fermentations by yeast and bacteria discussed up to this point deal with intramolecular processes, with the splitting of compounds in which the inner shifting of oxygen plays a part, then in the acetic fermentation an oxidative process is

displayed in which, beside a dehydrogenation the Cannizzaro rearrangement, was also supposed to take part. But the acetic fermentation is closely related to sugar splitting not only by the fact that many organisms can form acetic acid from carbohydrates but also by this fact, that in the oxidation of alcohol the substance which is formed by a definite agency is acted upon by another organism. Exactly as in the case of the previously described fixation of acetaldehyde as an intermediate product, so also in the case of acetic fermentation, especially by *B. ascendens* and *B. pasteurianum*, aldehyde, as the sulfite complex, accumulates in quantities which are quite appreciable. From one-third to three-fourths of the weight of acetic acid which is formed under the conditions of the experiment, is obtained as acetaldehyde.

Experience has shown that the fixation of acetaldehyde occurs much more satisfactorily in the presence of CaSO_3 than in the presence of Na_2SO_3 . The reason for this might be that the strongly alkaline alkali sulfite acts as a check on the agent causing the fermentation. Since the fixation of acetaldehyde can result only in accordance with an equilibrium—that in the case of acetic fermentation is displayed in the direction of dissociation—a part of the acetaldehyde is available for the normal process, i.e., for further transformations. This cannot be obtained directly, however, according to the most recent results, but under certain circumstances pretendedly in the manner of a Cannizzaro reaction on the acetaldehyde. If acetic acid bacteria are given pure acetaldehyde or pyruvic acid they always produce from it equi-molecular amounts of acetic acid and ethyl alcohol. This fact established a still closer parallelism between acetic fermentation and alcoholic fermentation.

According to the calculations of the energy conditions by V. Henri (48) an exothermic reaction is involved in the an-

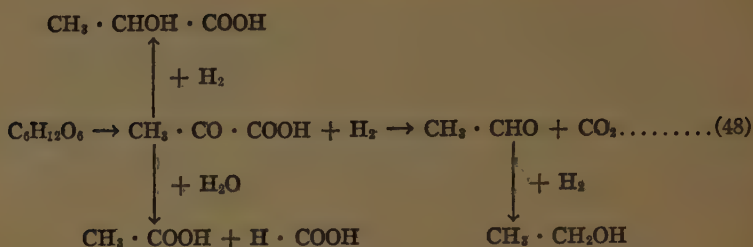
aerobic process. It might be desirable, however, to check the procedure of displacement or exclusion of air, under which conditions the foregoing experiments were carried on, since we know that the usual passing of a gas (or a mixture of gases) through a system of wash bottles does not secure a sufficient elimination of the oxygen admixed. The biological method of Beijerinck (9) on the other hand permits even the detection of 0.0007 per cent oxygen present in nitrogen. If namely this scruple should be proven as valid then it is possible to consider as explanation that the acetaldehyde may be also directly oxidized to acetic acid through dehydration of the water present. This would mean a plausible interpretation for this important reaction thus avoiding the necessity to consider further the highly improbable Cannizzaro reaction in this process.

The appearance of acetaldehyde in the fermentation of sugar by *B. lactis aerogenes* (125) or of pentoses by *B. acetoaethylicus* (148) and others, can also be demonstrated in like manner.

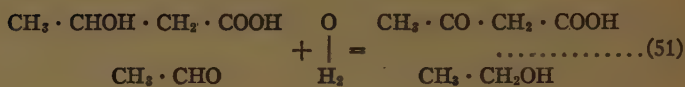
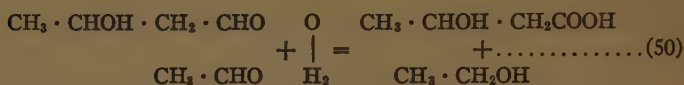
In this connection the recent work of Speakman (168) is of interest. The work which he began in 1914–1915 interprets in a convincing manner the mechanism of the acetone fermentation. In using *B. acetoaethylicus* he was able to demonstrate that in the fermentation of glucose, maltose or glycerol, pyruvic acid is the intermediate product which is the parent substance of the ethyl alcohol, as well as of the acetone, by the way of acetaldehyde.¹

At the onset of the fermentation, when practically no acetone is formed, the following formulas:

¹ Compare also Freiberg, G. W., Proc. Soc. Exp. Biol. and Med., 23, 72 (1925).

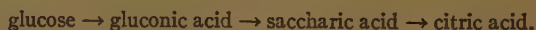


and later when rapidity of the hydrogenation of the acetaldehyde is decreased in relation to its formation, the following schemes,



explain the situation, as the alcohol: acetone proportion of 2.2 is established, beginning about the fifth day. It is attempted, as in the fermentation by yeast, to explain the processes through a joint reaction of different carbonyl compounds (see page 42).

It is quite important to note that recently the mechanism of the formation of citric acid from sugars has been cleared up by Walker, Subramaniam and Challenger (181) in so much as we have obtained experimental evidence for the first time that the conversion by means of *Aspergillus niger* proceeds according to the scheme:



This is a progress especially worthy of notice in view of the long preceding work in this field unsuccessfully attempting to show a detectable relation with the mechanism of sugar dissimilation by other cells.

The more our knowledge concerning the marvelous functions of the cell is increased, the more we appear to be justified in explaining chemical reactions on the generalization of a concept of transportation forms in contra-distinction to forms of a compound which can only be described by formulas represented in the usual manner of structural chemistry. However, before we can wholly explain the reactivity of the transportation form as contrasted with the ordinary form in its relation to the various variables, it would be necessary to know the rôle which the electrical forces² play in the activity of the transportation form or to investigate the physico-chemical conditions of the cell membrane.

² Compare J. N. Mukherjee and B. N. Ghosh, *J. Ind. Chem. Soc.*, 1, 213 (1924).

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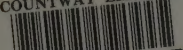
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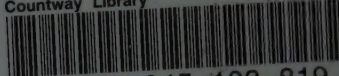
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